AUG 6 1923

THE JOURNAL

OF

EXPERIMENTAL MEDICINE

EDITED BY

SIMON FLEXNER, M.D. PEYTON ROUS, M.D.

VOLUME XXXVIII, No. 2 AUGUST 1, 1923

PUBLISHED MONTHLY

AT MOUNT ROYAL AND GUILFORD AVES., BALTIMORE, MD.

BY THE ROCKEFELLER INSTITUTE FOR MEDICAL RESEARCH

COPYRIGHT, 1923, BY THE ROCKEFELLER INSTITUTE FOR MEDICAL RESEARCH

Entered as second-class matter June 3, 1916, at the Post Office at Baltimore, Md., under the act of March 3, 1879.
Accepted for mailing at special rate of postage provided for in section 1103, act of October 3, 1917.
Authorized June 29, 1918.

Made in United States of America

PUBLICATIONS OF THE ROCKEFELLER INSTITUTE FOR MEDICAL RESEARCH

THE JOURNAL OF EXPERIMENTAL MEDICINE

EDITED BY

SIMON FLEXNER, M.D. PEYTON ROUS, M.D.

The Journal of Experimental Medicine is designed to cover the field of experimental medicine. Information regarding contributions and subscriptions is given in full on the back cover.

THE JOURNAL OF BIOLOGICAL CHEMISTRY

EDITED BY

STANLEY R. BENEDICT, New York, N. Y. LAFAYETTE B. MENDEL, New Haven, Conn.
H. D. DAKIN, Scarborough, N. Y. DONALD D. VAN SLYKE, New York, N. Y.

The Journal of Biological Chemistry is designed for the prompt publication of original investigations of a chemical nature in the biological sciences. It is issued monthly. Each volume consists of as many numbers as are required to make a total of approximately 800 pages. The subscription price is $5.00 per volume to subscribers in the United States and Canada, payable in advance; $5.25 to those in other countries. The number of volumes issued yearly averages four. The price of single copies varies; it is given on the back cover of each number. Remittances should be made by draft or check on New York, or by postal money order, payable to *The Journal of Biological Chemistry*, Mount Royal and Guilford Avenues, Baltimore, Md., or Avenue A and 66th Street, New York, N. Y.

THE JOURNAL OF GENERAL PHYSIOLOGY

EDITED BY

JACQUES LOEB, New York, N. Y. W. J. V. OSTERHOUT, Cambridge, Mass.

The Journal of General Physiology is devoted to the explanation of life phenomena on the basis of the physical and chemical constitution of living matter. It is issued bimonthly, one volume of about 600 pages appearing in a year. The subscription price per year (one volume) is $5.00, payable in advance. Single copies cost $1.00. Remittances should be made by draft or check on New York, or by postal money order, payable to *The Journal of General Physiology*, Mount Royal and Guilford Avenues, Baltimore, Md., or Avenue A and 66th Street, New York, N. Y.

STUDIES FROM THE ROCKEFELLER INSTITUTE FOR MEDICAL RESEARCH

The published results of investigations conducted in the Institute, or under its grants, are republished at irregular intervals in volumes designated *Studies from The Rockefeller Institute for Medical Research*. The subscription price is $2.00 per volume, payable in advance. A special rate of $1.00 per volume is made to those who subscribe to *The Journal of Experimental Medicine*, *The Journal of Biological Chemistry*, or *The Journal of General Physiology*. Remittances should be made by draft or check on New York, or by postal money order, payable to The Rockefeller Institute for Medical Research, Avenue A and 66th Street, New York, N. Y.

SEMIANNUAL LIST

The Institute publishes a *Semiannual List* of all papers by members of the staff and those working under grants from the Institute, stating the title and place of publication of these reports. The *Semiannual List* will be sent regularly upon application.

WAVERLY PRESS
WILLIAMS & WILKINS COMPANY
BALTIMORE, U. S. A.

THE PRESENCE OF BACTERIA IN THE LUNGS OF MICE FOLLOWING INHALATION.

By ERNEST G. STILLMAN, M.D.

(*From the Hospital of The Rockefeller Institute for Medical Research.*)

(Received for publication, March 13, 1923.)

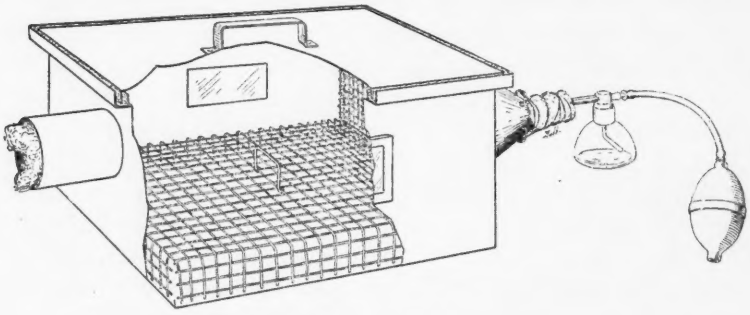
Considerable discussion has arisen at various times as to whether inspired bacteria reach the lungs or are filtered out in the nasal passages. After carefully reviewing the subject of elimination of bacteria from the respiratory tract, Bloomfield (1) concluded that in health and under average conditions of dust exposure microorganisms only occasionally penetrate below the larynx. He thinks that this result is chiefly due to the effective filtration system of the upper respiratory tract. Bacteria inspired into the upper respiratory tract, unless they incite disease, are eliminated by the ciliary currents, die *in situ*, or are carried off through the lymphatics. On the other hand, Jones (2) has recently shown that bacteria may be readily recovered from the borders of lungs removed from healthy calves, rabbits, guinea pigs, white rats, and mice. The microorganisms most frequently observed in cultures from the lungs of these normal animals were streptothrix, molds, and the bacteria of the *subtilis* group. Trillat and Kaneko (3) state that in mice, after 15 minutes of inhalation of a spray containing *Bacillus paratyphosus* and pneumococcus, these organisms are present in the pulmonary alveoli. They found that a much smaller number of paratyphoid bacilli were sufficient to cause infection in mice if they were administered by spraying than if they were mixed with the food or even injected subcutaneously. Wherry and Butterfield (4) have shown that pneumococci may be recovered from the deepest parts of the lungs of mice as long as 18 hours after spraying with a pneumococcus culture. Although they used a virulent Type I pneumococcus culture, none of the twenty-nine mice which were allowed to live, following the inhalation of the pneumococcus mist, became infected.

The purpose of the present study was to extend the above mentioned observations in the hope of obtaining further information concerning pulmonary infection under natural conditions.

Method.

Mice were used as the test animals. They were allowed to inhale an atmosphere in which a fine mist had been produced by spraying a culture of the organism to be studied. The organisms used were pneumococcus, *Streptococcus hæmolyticus*, *Bacillus influenzæ*, and staphylococcus.

The animals were placed in a galvanized iron box 12 by 10 by 7 inches (Text-fig. 1). This box was provided with two glass windows



TEXT-FIG. 1. Spray box.

in the sides and an air vent in one end which was plugged with cotton. At the opposite end was an opening through which the nozzle of an atomizer was inserted. The lid of the box rested in a cotton-filled groove. Before each experiment the entire box was sterilized in an autoclave. After the animals had been placed in the box, 10 to 15 cc. of broth culture were sprayed into the box with a hand atomizer, 10 to 15 minutes continuous spraying being sufficient to fill the interior of the box with a heavy cloud of finely divided droplets. As a rule, the mice were removed from the spray box 1 hour after spraying. At various intervals following the spraying, individual mice were killed and cultures were made from the lungs and other organs. In order to prevent the possibility of aspiration of bacteria from the upper to the

lower respiratory tract at the time of death, the mice were killed instantaneously by clamping the trachea and spinal cord with sponge forceps. The mice were then immersed in a solution of lysol and opened with sterile instruments. As a routine procedure, cultures in broth were made from the heart's blood, the spleen, and the lungs. In making the cultures pieces of the spleen and small pieces of the lung tissue from the borders of the lower lobes were placed directly in tubes of broth. All cultures which subsequently grew were plated on blood agar for further identification. All cultures were incubated

TABLE I.

Recovery of Pneumococci, Hemolytic Streptococci, Influenza Bacilli, and Staphylococci from the Lungs of Mice after Exposure to an Atmosphere Containing Culture in the Form of Spray.

Culture.	No. of mice exposed.	No. of mice killed.	Results of cultures from lungs. Length of time following exposure. 1 hr.	2 hrs.	3 hrs.	4 hrs.	5 hrs.	6 hrs.	8 hrs.	12 hrs.	24 hrs.	48 hrs.	No. of mice not killed.	No. of mice dying with septicemia.	No. of mice surviving.
Pneumococcus	12	6	+	+	−		−		−	−			6	1	5
Streptococcus hæmolyticus	12	6	+	+	+		+		+	+			6	6	0
B. influenzæ	10	6	+	−	+			−			+	−	4		4
Staphylococcus aureus	10	6	+		+	+		+			−	−	4		4

The pneumococcus and streptococcus cultures employed had a virulence such that 0.000001 cc. injected intraperitoneally into mice killed within 36 hours.

The cultures of *B. influenzæ* and *Staphylococcus aureus* had very little virulence on intraperitoneal injection.

for at least 36 hours. In a few instances growth was apparent only after 24 hours incubation. This was probably due to the small number of organisms present.

RESULTS.

In Table I is presented the protocol of one typical experiment. The total number of mice exposed to the atmosphere into which bacteria were sprayed was forty-four. Twelve of the mice were placed in a box into which a culture of pneumococcus was sprayed, twelve in a box into which a culture of *Streptococcus hæmolyticus* was

sprayed, ten in a box into which a culture of *Bacillus influenzæ* was sprayed, and ten in a box into which a culture of *Staphylococcus aureus* was sprayed.

Of the twelve mice exposed to the air containing pneumococci in suspension, six were killed at varying intervals of time, up to 12 hours. In cultures from the lungs of the mice killed 1 and 2 hours after exposure, pneumococci developed, while the cultures from the lungs of the mice killed after 3, 5, 8, and 12 hours remained sterile. Cultures from the spleen and from the heart's blood of all these mice remained sterile. The remaining six mice were not killed but were kept under observation. One died on the 3rd day. No gross lesions of the lungs were present, but cultures showed a general infection with pneumococcus. The other five mice survived and were discarded at the end of 14 days.

Of the twelve mice exposed to the air containing streptococci in suspension, six were killed at the end of 1, 2, 3, 5, 8, and 12 hours, respectively. From the lungs of all these mice streptococci were cultivated, while the cultures from the spleen and heart's blood remained sterile in all cases. Of the remaining six mice which were not killed, all died between the 5th and 10th days and all were found to have a general streptococcus infection.

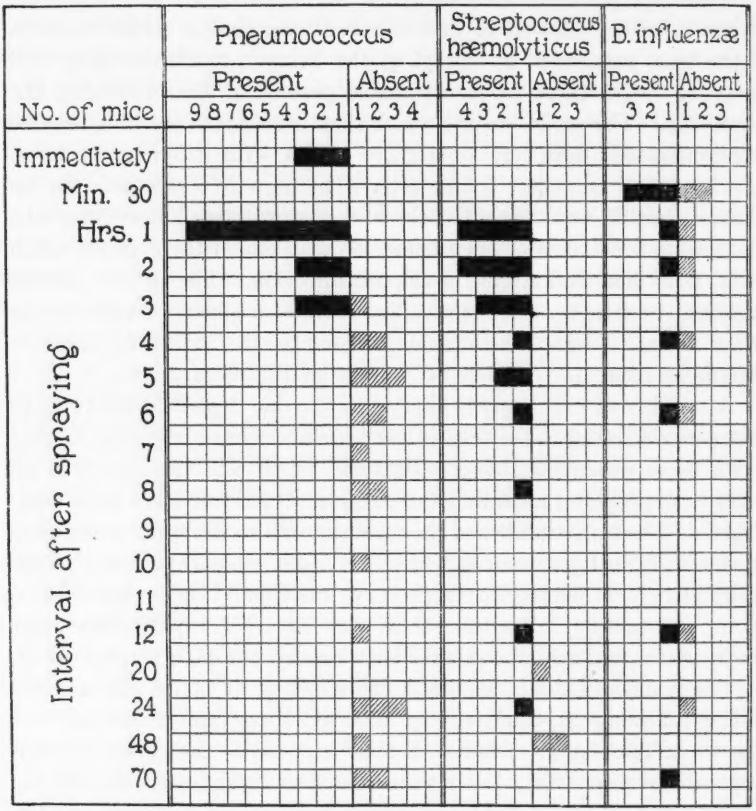
Ten mice were exposed to air containing influenza bacilli in suspension, and six of these were killed at the end of 1, 2, 3, 6, 24, and 48 hours, respectively. The results of the cultures from the lungs in these animals were irregular. From the lungs of the animals killed 1, 3, and 24 hours after spraying, influenza bacilli were cultivated, but the cultures from the lungs of those killed at the end of 2, 6, and 48 hours remained sterile. Cultures from the spleen and heart's blood remained sterile in all instances. The remaining four mice of this group were kept under observation for 10 days and all remained well.

Ten mice were exposed to air containing staphylococci in suspension. Six were killed at varying intervals, and from the lungs of the animals killed 1, 3, 4, and 6 hours after spraying, staphylococci were cultivated, while the cultures from the mice killed 24 and 48 hours after spraying remained sterile. All cultures from the heart's blood and spleen remained sterile. The remaining four mice of this group were not killed but were kept under observation for 10 days. All remained well.

This experiment demonstrates that under the conditions employed, when mice are allowed to breathe an atmosphere in which bacteria have been suspended by spraying, the bacteria reach the lungs and may be found there for considerable periods of time following the spraying. The period during which their presence in the lung may be demonstrated, however, apparently varies with different bacteria. Pneumococci were not found after 3 hours, while streptococci, influenza bacilli, and staphylococci were present after longer intervals. Following inhalation of pneumococcus, only one of the animals which were not killed died later of a general infection, while all the animals exposed to streptococcus spray died later of a general streptococcus septicemia. None of the animals exposed to the *Bacillus influenzæ* or staphylococcus spray suffered from a general fatal infection.

A number of experiments similar to the one described above have been carried out and the results in all instances have in general agreed with those described above. To avoid repetition, the results of all these experiments, including the one just described, have been combined. The total number of the mice exposed to the spray containing virulent Type I pneumococci was 228, those exposed to a spray containing virulent hemolytic streptococci was 45, and those exposed to a spray containing influenza bacilli was 32. No experiments other than that mentioned above have been carried out with staphylococci.

The results of these combined experiments, as far as the animals killed following exposure to the infected spray are concerned, are shown in Text-fig. 2. Of the 228 mice exposed to the spray containing pneumococci, thirty-seven were killed at varying intervals, and the results of the study of these thirty-seven mice are shown on the chart. It is seen that pneumococci were found to be present in the lungs of all of the fifteen mice killed within 2 hours of the exposure. Of four animals killed 3 hours after the exposure, pneumococci were demonstrated in the lungs in three instances, and the cultures from the lungs were negative in one. Of the eighteen mice killed after 3 hours, in the lungs of none could pneumococci be demonstrated. The remaining 191 mice of those exposed, were not killed but were kept under observation for at least 10 to 14 days. Twenty of these mice died, but in sixteen instances the cause of death was not due to pneumococcus infection, as in fourteen the cultures were sterile, and



TEXT-FIG. 2. Recovery of pneumococci, hemolytic streptococci, and influenza bacilli from the lungs of mice following spraying. Each square represents one mouse, the black squares indicating mice in which cultures of the lungs showed bacteria to be present, the cross-hatched squares representing mice in which cultures from the lungs remained sterile.

in two the cause of death was shown to be infection with a Gram-negative bacillus. The remaining four mice, however, died from a general pneumococcus infection, death in these instances occurring on the 3rd to 5th days following the exposure.

Of the forty-five mice exposed to the spray containing streptococci, twenty-one were killed, and the results of the cultures from the lungs in these mice are also shown in Text-fig. 2. It will be seen that of seventeen mice killed before the 12th hour following exposure, streptococci were demonstrated to be present in the lungs of all, and in one mouse killed after 24 hours streptococci were also cultivated from the lungs. In the three other mice, one killed after 20 hours and two after 48 hours, the cultures from the lungs remained sterile. The remaining twenty-four mice of the streptococcus series were not killed but were kept under observation. In striking contrast to the mice exposed to pneumococcus spray, seventeen, or 70 per cent, of these mice died between the 2nd and 12th days, and all suffered from a general streptococcus infection. Only seven mice were living at the end of 14 days.

Of the total thirty-two mice exposed to the spray containing influenza bacilli, seventeen were killed. Again the results of lung culture are shown in Text-fig. 2. It is obvious that here the results are irregular, in some instances the cultures from the lungs being positive and in others negative, both in animals killed soon after exposure and in others killed after much longer periods. The fifteen mice which were not killed were kept under observation for 14 days and none died during this period.

DISCUSSION.

The results obtained by combining all the experiments support the correctness of the previous observations. That during exposure of mice to a dense spray containing bacteria in suspension, the bacteria reach the lungs, seems to be demonstrated. The exact degree of penetration is as yet uncertain, but as the bacteria can be grown from very minute portions of lung tissue removed from the periphery, it seems safe to conclude that the bacteria reach the finer bronchi at least.

The further history of the bacteria which have penetrated the lungs seems to depend upon the variety of microorganism concerned. As regards pneumococcus, the bacteria are disposed of within a few hours. On the question as to whether they are killed *in situ*, are taken up by leucocytes, or are carried to other parts of the body, the present experiments throw no light. Streptococci, on the other hand,

at least with the cultures employed in these experiments, persist in the lungs for a much longer period of time. This fact may be associated with the occurrence of true infection, as subsequent events in many of these animals indicate. The results of the experiments in which *Bacillus influenzæ* was employed were more irregular; apparently sometimes these bacteria remain in the lungs for considerable periods of time, in other instances they disappear very quickly. Whether this result is associated with failure of the bacteria to penetrate deeply, or is related to the difficulty of cultivation of this organism, is not certain.

The fact that the penetration of the lungs by streptococci is usually followed by a general infection, while pneumococci are disposed of without any general infection resulting has been somewhat surprising. This was especially so in view of the fact that the virulence of the pneumococcus culture employed, as tested by intraperitoneal injection into mice, was equal to that of the streptococcus culture.

It was suggested that the apparent lack of invasiveness of the inhaled pneumococci might depend upon an insufficient number of organisms reaching the lung. That this view, however, is not tenable is shown by the following experiment. Six mice were exposed to an atmosphere containing pneumococci in suspension as previously described, and after 1 hour had elapsed all were killed. A single lobe of one lung was removed from each of the mice and each lobe was ground up separately in a mortar with sterile sand and normal salt solution. The supernatant fluid was then used for inoculation intraperitoneally into six normal mice. Five of the six mice injected died within 48 hours with a general pneumococcus infection. One mouse survived. It is evident, therefore, that in most instances pneumococci reach the lung in sufficient numbers to cause infection provided other conditions are favorable. It seems probable that normally the conditions in the lungs of mice are unfavorable for infection with pneumococci, even when the number of organisms reaching the lower respiratory tract is considerable. It is true that Blake and Cecil (5) infected monkeys with pneumococci by injecting minute amounts of the culture into the trachea, but even in these experiments a relatively considerable amount of fluid (1 cc.) was injected. The relation of the

injury produced by the penetration of the needle through the skin and tracheal mucous membrane to the subsequent infection should also be borne in mind.

In any case the present experiments indicate that in mice at least the mere presence of pneumococci in the lungs is not sufficient to induce infection, but that other factors are also concerned. An attempt has been made to discover something of the nature of these other factors by exposing the mice to altered external conditions before exposing them to the atmosphere of the spray box. The effects of sudden chilling and exposure to cold were studied. Mice were chilled before being placed in the spray box, others were kept chilled while they were in the box, and still others were chilled after the exposure in the box had taken place. They were chilled by being dipped into ice water, placed in an ice box, sprayed with cold water, or allowed to stand in melting snow. None of these procedures resulted in so changing conditions that infection occurred with any greater frequency than in the controls.

Attempts to lower local resistance by inducing slight injuries to the mucous membrane were also made. These attempts consisted in allowing animals to inhale ether before or after exposure to the spray, and certain mice were also exposed to an atmosphere containing finely divided quartz sand or talc in the form of dust before or following the exposure. These experiments also resulted in failure to increase the frequency of infection.

The present experiments, therefore, fail to shed any light on the exact mode of natural infection of the lung with pneumococci. They indicate that even in so susceptible an animal as the mouse other factors than the presence of the pneumococci in the lung are necessary for infection.

CONCLUSIONS.

1. When mice are exposed to an atmosphere containing cultures of bacteria in the form of a fine mist, the bacteria readily penetrate into the lower respiratory tract.

2. Pneumococci which have reached the lung as a result of this procedure usually disappear within a few hours and give rise to no

infection. Hemolytic streptococci, on the other hand, persist in the lung for a considerably longer time and a general septicemia usually follows.

3. Attempts to determine the conditions under which pneumococci which have reached the lung by inhalation may induce a local or general infection have not been successful.

BIBLIOGRAPHY.

1. Bloomfield, A. L., *Am. J. Med. Sc.*, 1922, clxiv, 854.
2. Jones, F. S., *J. Exp. Med.*, 1922, xxxvi, 317.
3. Trillat, A., and Kaneko, R., *Compt. rend. Acad.*, 1921, clxxiii, 109.
4. Wherry, W. B., and Butterfield, C. T., *J. Infect. Dis.*, 1920, xxvii, 315.
5. Blake, F. G., and Cecil, R. L., *J. Exp. Med.*, 1920, xxxi, 403.

PRODUCTION OF HETEROGENETIC ANTIBODIES WITH MIXTURES OF THE BINDING PART OF THE ANTIGEN AND PROTEIN.*

By K. LANDSTEINER, M.D., and S. SIMMS, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

(Received for publication, March 20, 1923.)

In 1911 Forssman[1] discovered the fact that rabbits injected with suspensions of organs of guinea pigs and a few other animals produce antibodies capable of hemolyzing sheep blood. These hemolysins differ from those which are produced in the usual way by injection of sheep erythrocytes. Because of their origin, they were called heterogenetic antibodies. Later the corresponding antigens (heterogenetic antigens) were found to be present in the tissues of many kinds of animals, but absent in others. Some facts relating to the properties of heterogenetic antigens that have a direct connection with our investigation are the following:[2]

Doerr and Pick[3] detected the resistance of the antigen to alcohol, and Sachs and Georgi[4] made use of alcoholic extracts for their experiments. Friedberger[5] (with Poor, Suto, and Schiff) found the heterogenetic antigen present in the urine of animals to be soluble in alcohol (Doerr and Pick). Sordelli and his coworkers[6] pointed out that the extracts have not only, as known before, the property of combining with the corresponding antibodies but can be flocculated by the immune sera (*cf.* Sachs and Guth[7]). They stated that the extracts are devoid of any power to produce antibodies when injected into rabbits. On the other hand, these authors claimed that heterogenetic antibodies can be produced by injection of the alcohol-

* Sixteenth paper on antigens.

[1] Forssman, J., *Biochem. Z.*, 1911, xxxvii, 78.

[2] For a more complete quotation of the literature, consult the articles mentioned.

[3] Doerr, R., and Pick, R., *Biochem. Z.*, 1913, l, 129.

[4] Sachs, H., and Georgi, W., *Z. Immunitätsforsch., Orig.*, 1914, xxi, 346. *Cf.* Georgi, W., *Arb. k. Inst. exp. Therap. Frankf.*, 1919–20, No. 9, 43.

[5] Friedberger, E., and Suto, K., *Z. Immunitätsforsch., Orig.*, 1919, xxviii, 217.

[6] Sordelli, A., and coworkers, *Rev. inst. bact. Buenos Aires*, 1918, i.

[7] Sachs, H., and Guth, F., *Med. Klin.*, 1920, xvi, 157.

insoluble residue, in spite of its inactivity against the antibodies *in vitro*. In a later review[8] of their work the authors repeated this statement, adding that the antigenic activity of the residue is weak. Landsteiner[9] obtained similar results, but laid stress on the fact that the antigenic power of the residue after alcohol extraction is markedly diminished. Furthermore, it is completely, or almost completely destroyed by treating the material (horse kidney) for a short time with boiling alcohol.

One of the present authors has tried to explain the known facts by the assumption that the heterogenetic antigens consist of two parts, combined with each other and capable of being separated by the action of alcohol. One part is a protein and is necessary for the production of antibodies. The other, which is alcohol-soluble and perhaps a lipoid, contains the specific group but possesses no marked antigenic property and is active as an antigen when combined with proteins. This view was strengthened by experiments[10] which demonstrated that substances of a simple chemical constitution form antigens in combination with proteins and that the corresponding antibodies act specifically on these simple substances. Owing to the lack of a suitable term for such bodies, hitherto but not quite correctly spoken of as antigens, the term haptene was proposed in our previous communication. This term designates, therefore, substances which, while acting strongly and specifically with homologous antibodies, have little or no antigenic properties as compared with the binding power, but become antigens when combined with proteins. It is possible that there exist a number of such substances, as, for example, the alcohol-soluble products studied by K. Meyer[11] and Waelsch, and perhaps the bacterial substances described by Dochez and Avery,[12] Zinsser and Parker,[13] and Heidelberger and Avery.[14] It is still

[8] Sordelli, A., and Fischer, G., *Compt. rend. Soc. biol.*, 1921, lxxxiv, 174.

[9] Landsteiner, K., *Proc. Acad. Sc. Amsterdam*, February, 1921, xxiii, 1166; *Biochem. Z.*, 1921, cxix, 294.

[10] Landsteiner, K., *Biochem. Z.*, 1919, xciii, 106; 1920, civ, 280.

[11] Meyer, K., *Z. Immunitätsforsch., Orig.*, 1910, vii; 1911, ix; xi; 1922, xxxiv.

[12] Dochez, A. R., and Avery, O. T., *J. Exp. Med.*, 1917, xxvi, 477.

[13] Zinsser, H., *J. Exp. Med.*, 1921, xxxiv, 495. Zinsser, H., and Parker, J. T., *J. Exp. Med.*, 1923, xxxvii, 275.

[14] Heidelberger, M., and Avery, O. T., *J. Exp. Med.*, 1923, xxxviii, 73. Avery, O. T., and Heidelberger, M., *J. Exp. Med.*, 1923, xxxviii, 81.

questionable whether the so called Wassermann antigen has any relation to the present subject (see Seligmann[15]).

The foregoing hypothesis on the nature of the heterogenetic antigen was afterwards independently put forward by Taniguchi[16] and von Gutfeld[17] (see Doerr[18]). In view of the new facts and their interpretation, the existence of nearly related antigens in the tissues of non-related animals appears less surprising than it was previously thought. Such an occurrence would simply depend on the presence of one substance (or several similar substances) of comparatively simple constitution in all these tissues. With regard to the chemical nature of the haptene, according to K. Meyer,[19] it belongs to the group of lecithins and cephalins, while Wernicke and Sordelli[20] designate it as a cerebroside. We believe, however, that the facts put forward up to the present are not yet sufficient to warrant any definite statement as to the purity of the preparations or their precise chemical constitution, especially when one considers the intricacies of the chemical investigation of lipoids.

In order to test the hypothesis above mentioned, we tried to produce antigenic effects by combining the haptenes artificially with proteins—as it were, to synthesize the antigen. The result could not be predicted, as in our previous experiments new antigens were built up from chemical substances which could unite with proteins by means of definite chemical reactions. Such a reaction not being at our disposal in the present investigation, we tried to attain our object by simply mixing the alcoholic extracts with protein-containing solutions. It was thought that in this way a loose (adsorption) compound might be formed.[21] It may be recalled that the natural heterogenetic antigen is also, according to our opinion, a loose compound which can be split up by simple treatment with alcohol.[22]

[15] Seligmann, E., and Pinkus, F., *Z. Immunitätsforsch., Orig.*, 1910, v, 377.

[16] Taniguchi, T., *J. Path. and Bact.*, 1921, xxiv, 253, 254.

[17] von Gutfeld, F., *Z. Immunitätsforsch., Orig.*, 1922, xxxiv, 524.

[18] Doerr, R., *Ergebn. Hyg.*, 1922, v, 168, 130.

[19] Meyer, K., *Biochem. Z.*, 1921, cxxii, 225.

[20] Wernicke, R., and Sordelli, A., *Compt. rend. Soc. biol.*, 1921, No. 3; *Rev. inst. bact. Buenos Aires*, 1919, ii, 281.

[21] It is perhaps possible that the natural hemolysins which are present in the sera employed play a part in the formation of the supposed compounds.

[22] One of the experiments has been briefly reported previously (Landsteiner, K., *Biochem. Z.*, 1921, cxix, 306; *Proc. Acad. Sc. Amsterdam*, 1922, xxiv, 237).

EXPERIMENTAL.

For the injections rabbits were used. To obtain reliable results it was necessary to treat the different series of animals in the same way and at the same time and to use a sufficient number for each experiment. For although the action of heterogenetic antigen is remarkably constant (Doerr) the difference in the reaction of individual animals is great enough to mask the underlying principles when only a small number of animals are studied. Furthermore, in selecting the animals those were excluded in which the serum, prior to the injection, gave a strong or complete hemolysis in a dilution of 1:25 in half an hour under the conditions of the tests. In this way the initial hemolytic activity of the sera was rendered negligible. This method was considered better than to employ rabbits with hemolyzing sera and to calculate the ratio between the initial and the final values, because it is not known whether the production of antibodies in immunized animals is proportional to the initial amount of normal antibodies, other conditions being equal.

Preparation of the Material for Injection.—150 gm. of horse kidney were passed through a mincing machine, sometimes through a sieve. To the pulp were added 750 cc. of 95 per cent alcohol and the suspension was kept at room temperature for 2 days with occasional shaking. The suspension was then filtered and the insoluble residue similarly treated with 450 cc. of 95 per cent alcohol. The first extract was evaporated almost to dryness on a water bath, then dissolved in the filtrate of the second extraction by heat, filtered when hot, and evaporated nearly to dryness. The residue was emulsified in 50 cc. of 0.9 per cent saline.

From this stock solution the injection solutions were prepared by diluting twenty times with 0.9 per cent saline or in the same proportion with diluted serum. The saline emulsions and the mixtures with serum thus contained the same percentage of kidney extract. The sera (human or pig) were diluted eight to ten times with 0.9 per cent saline. To all solutions 0.25 per cent phenol was added. A sufficient quantity of the solutions was made for each series of injections and preserved in the ice box.

Preparation of Antibodies.—The rabbits, fed with vegetables and oats, were injected with 5 cc. of solution each time, intravenously or intraperitoneally, the injections being repeated five to six times at intervals of 7 to 10 days.

Tests for Hemolysis.—The sera of the rabbits were taken 7 days after the last injection. To 0.5 cc. of dilutions of each serum, 0.5 cc. of fresh guinea pig serum, diluted 1:10, was added which itself was not hemolytic under the conditions of the experiment. 1 drop (about 0.05 cc.) of 50 per cent washed sheep erythrocytes was added and the tubes were incubated at 37°C.

I. Injections of Mixtures of Extract and Serum.

Experiment 1 (*Table I*).—Materials injected into various batches of rabbits: (*a*) pig serum 1:10; (*b*) extract of horse kidney; (*c*) the same as (*a*), heated for ½ hour at 80°C.; (*d*) mixture of horse kidney extract and pig serum 1:10; (*e*) the same as (*d*), heated for ½ hour at 80°C. Tests after six intraperitoneal injections. Dilution of the rabbit sera 1:250. Period of incubation 1 hour.

TABLE I.

Material injected.	Pig serum (*a*).				Kidney extract (*b*).				Heated pig serum (*c*).			Mixture of kidney extract and pig serum (*d*).				Mixture of kidney extract and pig serum heated (*e*).			
Rabbit No.....	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
Hemolysis.	0	0	0	F.Tr.	0	0	0	Tr.	0	0	0	C.	M.	Sl.	F.Tr.	Tr.	St.	Sl.	A.C.

In the tables C. indicates complete hemolysis; A.C., almost complete; V.St., very strong; St., strong; M., marked; Sl., slight; Tr., trace; F.Tr., faint trace; and 0, no hemolysis.

The experiment shows a marked difference in the response to the extract and to the mixture of extract and serum. There is no distinct difference between that to the heated and unheated mixtures. No hemolytic power of the sera was found after injections of pig serum in the dilution used.

Experiment 2.—Similar results were obtained after four intraperitoneal injections into three rabbits of alcoholic extract mixed with pig serum diluted 2.5 times. In this instance the mixture was faintly acidified with acetic acid and coagulated by heating for ½ hour to 75-80°C.

Experiment 3 (*Table II*).—Materials injected: (*a*) pig serum diluted 1:8; (*b*) mixture of kidney extract and 1:8 diluted pig serum. Tests after four intravenous injections. Dilution of the sera 1:250. Period of incubation 1 hour.

TABLE II.

Material injected.	Pig serum (*a*).				Mixture of kidney extract and pig serum (*b*).		
Rabbit No.....	23	24	25	26	27	28	29
Hemolysis.....	0	0	Tr.	0	0	C.	A.C.

As part of the same experiment four rabbits were injected subcutaneously with the mixture. The sera of these animals never manifested more than a slight hemolytic activity.

Experiment 4 (*Table III*).—Materials injected: (*a*) human serum diluted 1:8; (*b*) extract of horse kidney; (*c*) mixture of horse kidney and human serum diluted 1:8. Tests were made after five intravenous injections. Dilutions of the sera 1:500. Period of incubation 1 hour.

TABLE III.

Material injected	Human serum (*a*).											
Rabbit No.	34		35		36		37		38			
Hemolysis	0		Tr.		0		0		Tr.			
Material injected	Kidney extract (*b*).											
Rabbit No.	39	40	41	42	43	44	45	46	47	48	49	50
Hemolysis	0	Tr.	0	0	0	Sl.	0	Tr.	0	0	A.C.	0
Material injected	Mixture of kidney extract and human serum (*c*).											
Rabbit No.	51	52	53	54	55	56	57	58	59	60	61	62
Hemolysis	C.	C.	Sl.	C.	A.C.	C.	Tr.	C.	C.	C.	C.	C.

The dilutions up to which complete hemolysis took place are given in Table IV. According to these results the average titer is about eighteen times higher in the second series than in the first. Moreover, the experiment clearly shows some increase of the hemolytic power in a number of the animals injected with the extract alone. In only one of them is the titer remarkably high (No. 49), but in another experiment, not quoted here, two out of five animals showed a similar increase and one a still higher hemolytic activity. It should be mentioned that in these particular animals coccidiosis was found. It is uncertain whether or not this observation is of any significance.

With regard to the action of the extract alone, an opportunity was afforded in Experiment 4 of comparing in different stages of immunization the sera of rabbits injected with alcoholic extract. The tests were performed at the same time, one set of animals having received two, the other four injections. The dilution of the sera was 1:25, the incubation period 15 minutes. The results are given in Table V.

In addition to the hemolytic activity of the sera, the precipitating power was also tested in some of the experiments in which proteins were injected. It was found that frequently the most active hemolysins and precipitins were developed in the same animals, but this parallelism was not constant.

TABLE IV.

Material injected	Kidney extract (*b*).											
Rabbit No.	39	40	41	42	43	44	45	46	47	48	49	50
Complete hemolysis up to	1:<25	1:50	1:<25	1:<25	1:25	1:100	1:<25	1:100	1:25	1:<25	1:250	1:<25
Material injected	Mixture of kidney extract and human serum (*c*).											
Rabbit No.	51	52	53	54	55	56	57	58	59	60	61	62
Complete hemolysis up to	1:1,000	1:1,000	1:100	1:2,000	1:250	1:1,000	1:50	1:2,000	1:1,000	1:2,000	1:1,000	1:1,000

TABLE V.

No. of injections with kidney extract.	Two.						Four.					
Hemolysis	0	Tr.	0	0	Sl.	0	M.	A.C.	St.	0	St.	C.

II. Comparison between Injections of Mixtures and Separate Injections of the Components.

Although a definite increase of the hemolytic activity was not found after injection of either pig or human serum, the possibility had to be borne in mind that by injecting two weakly active substances; *i.e.* extract + serum, instead of a single one a summation of the effects could result. Therefore in the following experiments two series of rabbits were injected. The animals of the first series received 5 cc. of the mixture intravenously as in the preceding experiments. The animals of the second series received 5 cc. of the emulsion of kidney extract into one ear vein and 5 cc. of the diluted serum into the ear vein of the other side. Thus each animal of the two series received the same quantity of both substances, and if the effect had been due simply to an addition, both groups should have reacted in the same degree. This experiment is also related to another point. One might suppose that if a non-antigenic substance is injected into an animal during the period of immunization brought about by an antigen, the former substance would be included, as it were, in the process of immunization. The results obtained, however, do not support such a view.

Experiment 5 (*Table VI*).—Materials injected: pig serum diluted 1:8; and kidney extract. Tests after five intravenous injections. Rabbit sera diluted 1:500. Period of incubation 1 hour.

TABLE VI.

	Separate injections of extract and serum.						
Rabbit No.	63	64	65	66	67	68	69
Hemolysis	0	0	0	V.St.	Sl.	C.	0
Complete hemolysis up to						1:500	

	Mixture.								
Rabbit No.	70	71	72	73	74	75	76	77	78
Hemolysis	C.	V.St.	V.St.	A.C.	A.C.	C.	V.St.	C.	C.
Complete hemolysis up to	1:2,000					1:500		1:1,000	1:500

Experiment 6 (*Table VII*).—Materials injected: human serum diluted 1:8; and kidney extract. In addition to the injections of both components and the mix-

ture, a third series of five rabbits was injected intravenously with diluted human serum alone. Tests after five intravenous injections. Rabbit sera diluted 1:500. Incubation period 15 minutes and 1 hour.

In these two experiments the difference between the two sets of animals in which the same substances were injected in equal quantities but in different ways is very marked. The question as to whether injections of extract and serum made separately are more effective than the injections of extract alone has not yet been studied sufficiently to venture any statement.

TABLE VII.

	Human serum.				
Rabbit No.	79	80	81	82	83
Hemolysis after 1 hr.	0	0	0	0	0

	Separate injections of extract and serum.						
Rabbit No.	84	85	86	87	88	89	90
Hemolysis after 15 min.	0	0	0	0	0	M.	0
" " 1 hr.	0	Tr.	Tr.	0	F.Tr.	A.C.	M.

	Mixture.						
Rabbit No.	91	92	93	94	95	96	97
Hemolysis after 15 min.	C.	M.	V.St.	Sl.	A.C.	V.St.	0
" " 1 hr.	C.	A.C.	C.	V.St.	C.	C.	M.
Complete hemolysis up to	1:2,000		1:1,000		1:1,000	1:1,000	

DISCUSSION.

As the preceding experiments show, the antigenic power of heterogenetic antigen destroyed by the action of alcohol can be restored to a considerable extent by admixture of protein solutions such as serum. This effect can be demonstrated relatively easily, as the response of animals to heterogenetic antigen is more regular than with most other antigens, so that when the native antigen is used, antibodies can be obtained from almost every injected rabbit. However, the action of the artificial combination is not equal to that of the native antigen and more intensive treatment is required to produce the same result. Yet in this manner a satisfactory hemolytic titer is frequently obtained.

The related observations thus support the conception that the heterogenetic antigen is composed of two parts, as suggested above.

The supposition that a combination of the alcohol-soluble haptene with the added protein is, in our experiments, the acting substance seems to be the simplest interpretation of the results. The difference in the activity of the sera, when the mixture of haptene and protein is injected as such, or each component separately, demonstrates that some change results from mixing the two substances *in vitro*. The assumption that the effect of the injected mixture is due to some modification other than the formation of a compound seems hardly probable, since a mixture of the haptene with serum also occurs when haptene alone is injected intravenously. In the latter case, however, no marked effect follows. From our point of view this result is comprehensible because homologous proteins are much less adapted to antigenic action than foreign ones. They doubtless can be transformed into antigens[23] even by comparatively slight changes but are always inferior to materials of foreign origin. If doubt remain in the matter it could probably be tested by injections of the heterogenetic haptene mixed with rabbit serum.

With regard to the alcohol-soluble body itself, it is now almost generally admitted[24] that it has no antigenic power. This statement is approximately but not absolutely correct.[25] Observations on a considerable number of repeatedly injected rabbits leave no doubt as to the occurrence of a slight increase in hemolytic power in some of them, even if the few exceptional cases of high titer are ruled out. This finding can be attributed either to real immunization or to an enhanced output of normal antibodies, but the first mentioned explanation seems to be far more likely. There is reason then to believe that after injection of non-protein substances production of antibodies can take place,[26] if to but a slight degree. One can suppose that such an immun-

[23] Pick, R., and Obermayer, F. Landsteiner, K., and Jablons, B., *Z. Immunitätsforsch., Orig.*, 1913–14, xx, 618. Landsteiner, K., and Lampl, H., *Z. Immunitätsforsch., Orig.*, 1917, xxvi, 293.

[24] Sordelli and coworkers, Taniguchi, and von Gutfeld. *Cf.* Meyer, K., *Biochem. Z.*, 1921, cxxii, 225.

[25] *Cf.* Landsteiner, K., *Biochem. Z.*, 1921, cxix, 298, 304.

[26] K. Meyer, Kleinschmidt, Much, and others.

ization is due to the formation of compounds of the haptene with proteins of the rabbit, as in the experiments cited above, and a similar explanation may hold true for the other instances in which antigenic power is ascribed to lipoids, so called. As this view is only hypothetical, it does not enable one to make a sharp distinction between antigenic and non-antigenic substances. But in practise some discrimination is possible because of the wide differences in the activity of the two classes. The immunizing effects ascribed to non-protein substances, as, for example, fats or lipoids, are slight and irregular, and the only substances which can be used effectively for the preparation of antibodies are proteins, if bodies of unknown chemical nature such as toxins are excluded.[27] Consequently it may be assumed that a somewhat different mechanism is concerned in the two instances mentioned. In any case there would appear to be an intimate connection of proteins with the process of antibody production, which is probably not only dependent on the size of the molecules[28] but also on the chemical structure.

The fact that the addition of protein to a substance can transform it into an efficient antigen suggests similar investigation of other substances.[29]

CONCLUSIONS.

1. The alcohol-soluble extract of heterogenetic antigen, which possesses the specific chemical structure of the entire antigen, has a detectable but generally very slight power to increase the amount of heterogenetic antibodies, when injected into rabbits.

2. This substance can be transformed into an efficient antigen by mixing it with protein solutions such as diluted normal serum.

3. Such mixtures are considerably more active than the same substances injected separately. Therefore, the effect of the serum is probably due to the formation *in vitro* of a loose compound between the

[27] See Wells, H. G., Chemical pathology, Philadelphia and London, 2nd edition, 1914.

[28] Landsteiner, K., *Biochem. Z.*, 1919, xciii, 106.

[29] *Cf.* Doerr, R., *Schweiz. med. Woch.*, 1921, ii, 937; Schnabel, A., *Jahresk. ärztl. Fortbild.*, 1920, xi, 15.

alcohol-soluble substance and protein, the compound acting as a complete antigen.

4. It may be supposed that there exists a group of natural antigens which are built up of one specifically reacting part that is almost or entirely devoid of antigenic properties, and another part, a protein, responsible for the immunizing effect.

AN INFECTIOUS OPHTHALMIA OF CATTLE.

By F. S. JONES, V.M.D., and RALPH B. LITTLE, V.M.D.

(*From the Department of Animal Pathology of The Rockefeller Institute for Medical Research, Princeton, N. J.*)

PLATE 12.

(Received for publication, March 23, 1923.)

During the months of September and October, 1922, there occurred among cattle recently introduced into a dairy herd a considerable number of cases of ophthalmia. Inasmuch as cases of pneumonia associated with *Bacillus bovisepticus* appeared among the animals at the same time the two diseases were confused. At first it was thought that the ophthalmia might be a local manifestation of the infection with *Bacillus bovisepticus*. Among the first cases examined were several which showed unmistakable evidence of respiratory diseases as well as acute ophthalmia. The results of bacteriological examination soon eliminated *Bacillus bovisepticus* as the possible etiological agent.

The disease was introduced among cows recently purchased from a dealer in Ohio. Several were suffering from ophthalmia when received. These animals were housed in a barn with two other lots of newly purchased cows. Eleven of these subsequently became infected. Six other cows in an adjoining barn also developed the same disease. In all, it was possible to isolate a characteristic organism from twenty-four cases.

Clinical Characters.

The infection was characterized by its sudden onset. There was marked photophobia and impairment of vision. Lacrimation was excessive and accompanied by considerable thick, yellow discharge which gathered at the inner canthus. The exudate often washed downwards soiling the hairs of the face (Fig. 1). The eyelids were thickened. Palpation of the lids caused considerable discomfort.

The membrana nictitans was often deeply reddened and swollen. The conjunctiva was bright red in color. The vessels of the eyeball were injected. Fine strings of the yellowish white exudate were often observed on the eyeball or conjunctiva. In a few instances shallow, irregular ulcers were encountered on the cornea. In three cases there was extensive corneal opacity. Here interference with vision was severe. It was feared that the sight would be lost, but the opacities became smaller and finally disappeared. In certain cows but one eye was affected, in others both eyes were involved.

Bacteriology.

Fresh exudate was obtained from the inner canthus and beneath the lids on sterile cotton swabs. Films were prepared on sterile cover-slips. The remaining material was suspended in salt solution and blood agar plates prepared at once. At first the swabs were smeared directly over slants of various media but the usual presence of spreading colonies of *Bacillus subtilis*, molds, etc., frequently made identification difficult. On the whole, when the organisms were numerous the plates from the salt solution suspensions were more satisfactory. Films of the exudate when stained with methylene blue or other aqueous stains usually revealed long strands of mucus. In certain cases the exudate was apparently composed only of mucus (Fig. 2) with a few epithelial cells. In others the exudate contained leucocytes in large numbers (Fig. 3). Exudates when stained by Weigert's method often showed the presence of fibrin in small amounts. The organisms were usually abundant. The bacterium most frequently observed was a short rod with rounded ends. It usually occurred in pairs. Morphologically it appeared indistinguishable from published figures of the Morax[1]-Axenfeld[2] diplobacillus of human pink-eye. At first it was regarded as Gram-positive. After staining with Stirling's gentian violet and fixing with Lugol's solution it resisted decolorization with alcohol to a considerable degree but eventually became decolorized.

[1] Morax, V., *Ann. Inst. Pasteur*, 1896, x, 337.
[2] Axenfeld, T., *Centr. Bakt., Ite Abt.*, 1897, xxi, 1.

Morphological and Cultural Characters of the Diplobacillus.

Morphology.—The individual elements are short, plump bacilli with rounded ends. The length is fairly uniform in 24 or 48 hour cultures, varying from 1.5 to 2 microns, and the width is 0.5 micron. Longer forms are not infrequent in the exudate. The bacilli usually occur in pairs and short chains both in the exudate and in culture media. Chains of four to eight are common in bouillon. It is non-motile and does not form spores. Well developed capsules may be demonstrated in suitable preparations. The organism stains well by ordinary methods and it is Gram-negative. Growth is better on media containing blood or serum.

Colonies.—In agar plates containing 8 per cent of defibrinated horse blood the colonies are characteristic. After 24 hours the surface colonies are round, translucent, and grayish white. They are surrounded by a narrow, clear zone of hemolysis. The deep colonies are at this time barely perceptible to the naked eye. In sharp contrast is their clear hemolytic zone, usually 1.5 mm. in diameter. After 48 hours the surface colonies are more flattened in appearance and may reach a diameter of 3.5 to 4 mm. (Fig. 4). The deep colonies are ovoid and biconvex in shape. The hemolytic area has increased in diameter to 2.5 to 3 mm.

Blood Agar Slants.—After 24 hours at 38°C. there is a heavy, viscid, grayish white growth which does not tend to spread on longer incubation. The blood in the condensation water is hemolyzed.

Plain and Serum Agar Slants.—These reveal a similar growth but more restricted than that on blood agar.

Coagulated Serum.—On the surface there appear after 18 hours tiny pits which gradually enlarge as incubation continues into saucer-shaped centers of liquefaction. Later the centers of liquefaction coalesce and after 10 days about two-thirds of the medium is liquefied.

Agar and Serum Agar Stab.—The growth is confined to the surface or 1 or 2 mm. along the needle track.

Gelatin.—At 22°C., growth is slow. At first the surface is pitted like the coagulated serum. Within 7 days there is distinct liquefaction which progresses very slowly.

Plain Bouillon.—Growth takes place slowly. There is a slight turbidity with considerable sediment. On agitation the sediment breaks up into coarse particles.

Dextrose Bouillon in Fermentation Tubes.—The growth is confined to the bulb and resembles that in plain bouillon. There is no fermentation. The medium tends to become more alkaline.

Litmus Milk.—The medium becomes more alkaline. After 10 days three distinct zones are visible: an upper, liquid zone of a deep blue color, an intermediate zone lighter in color and containing soft flocculi of casein, and at the bottom of the tube a still lighter portion of coagulated casein.

No growth has been obtained on potato. Acid production has not been noted in any of the carbohydrate media usually employed. Indole is not found in sugar-free broth after 14 days incubation. A temperature of 58–59°C. for 5 minutes kills the organism. When the growth from 24 hour blood agar slants is smeared on sterile cover-slips and dried at room temperature the organism remains viable for 3 days. After this time growth can no longer be obtained.

Pathogenicity for Small Animals.

White mice and rabbits are refractory to subcutaneous or intraperitoneal injection of suspensions of exudate made from the eyes of cattle. In three instances a little of the suspension was dropped into the eyes of normal rabbits. The animals failed to react. Guinea pigs and rabbits injected intraperitoneally with heavy suspensions from freshly isolated cultures remained well. Heavy suspensions of cultures dropped into the eyes of rabbits and guinea pigs failed to produce noticeable effects.

EXPERIMENTAL.

Although the evidence pointed to the diplobacillus as the etiological agent there still existed some doubt as to the possible existence of some underlying agent. It was decided to inoculate fresh suspensions of exudate obtained from acute cases with the hope that if other organisms were responsible they might be detected early in the course of the infection.

With this in view a young calf was first inoculated by dropping a little of the fresh suspension on the eyeball and retaining it beneath the lower lid. The animal developed a mild inflammation of the eyes 2 days later, from which the diplobacilli were recovered. On the whole, the disease produced was so mild that it might have been due to mechanical causes.

A 6 months old bull was subsequently inoculated in the right eye with 0.5 cc. of a suspension of the exudate from two cases. The following protocol gives the results in some detail.

Bull Calf 898.—On Sept. 16, 1922, 0.5 cc. of a salt solution suspension of exudate from two acute cases was dropped on the eyeball and retained for a few seconds beneath the lower lid of the right eye. Sept. 17 to 19. The eye appeared normal. On Sept. 20, a ramiform congestion of the vessels of the eyeball appeared. The membrana nictitans was swollen and of a bright pink color. The conjunctiva was deeply reddened. The eyes were sensitive to light. There was considerable thick, yellowish white discharge at the inner canthus. This on microscopic examination was mucopurulent. Diplobacilli were present in considerable numbers. Plate cultures revealed a few colonies of streptococci and many colonies of diplobacilli. The discharge continued for 3 or 4 days and gradually became less. The diplobacilli were observed in the films and were cultivated in plate cultures. On Sept. 28, the left eye became involved. A large amount of yellowish white exudate was observed at the inner canthus. The membrana nictitans was swollen and red. Microscopic examination of films from the exudate revealed strings of mucus, leucocytes, and a moderate number of diplobacilli. Over 80 per cent of the colonies which developed in the plate cultures were of the characteristic hemolytic type. The inflammation in the other eye was more pronounced at this time. The reaction gradually subsided and within 10 days disappeared. On Oct. 20, the eyes appeared normal, except for a trace of discharge at the inner corner of the right. Films prepared from both eyes still showed diplobacilli. The organisms were numerous and made up 95 per cent of the colonies developing in the plate cultures. Treatments three times a day with a 1:40 solution of zinc sulfate were begun on Oct. 23. A small quantity of the solution was dropped on the eyeball and retained for a brief period beneath the lids. On Oct. 26, the organisms could not be found in films. Plate cultures were also negative. The treatment was continued until Oct. 28. On this day material was again examined with negative results.

The preceding experiment strongly indicated that the diplobacilli were responsible for the disease. Streptococci in small numbers were also present but the diplobacilli made up from 70 to 95 per cent of the organisms present. Their persistence after the acute symptoms had subsided was significant. To establish definitely the etiological

relationship of this diplobacillus pure cultures were dropped in the eyes of two cattle. A detailed report of one case is given.

Cow 897.—On Oct. 10, a Holstein cow that had been under observation for several months was isolated in a tightly screened unit. On this day one-half the growth from pure cultures isolated from two spontaneous cases were each suspended in 2 cc. of sterile bouillon. 6 drops of these suspensions were permitted to run over the eyeballs. The right eye received culture from Cow 4301, the left the suspension from Culture 4316. On Oct. 11, lacrimation was pronounced. On Oct. 12, the eyes appeared normal, except for barely discernible swelling about the lids at the median borders. On Oct. 13, the lids of the right eye were appreciably thickened. The conjunctiva was reddened. The vessels of the eyeball were injected. A small amount of yellowish white, thick discharge was present at the inner canthus. Shreds of exudate were observed on the surface of the eyeball. The condition of the left eye was about the same, but the discharge was more copious. Microscopic examination of exudate from the right eye revealed desquamated epithelial cells, mucus, and large numbers of diplobacilli. Material from the left eye showed polymorphonuclear leucocytes in addition to epithelial cells, mucus, and diplobacilli (Fig. 5). The plate cultures made from salt solution suspensions of exudate developed the hemolytic colonies in practically pure cultures. Observations were made daily. The ophthalmia became more pronounced during the next few days. On Oct. 15, swelling of the lids was pronounced. It extended for a distance of 5 cm. beyond the borders of the lids. About the inner corners of the eye the characteristic yellowish white exudate was observed. Palpation caused considerable discomfort. Congestion of the conjunctiva was pronounced. On Oct. 19, the manifestations of inflammation were severe. On Oct. 23, edema of the lids and congestion of the conjunctiva were accompanied by a mucopurulent exudate. The diplobacillus was present in the films and plate cultures in enormous numbers. Treatment with a 1:40 solution of zinc sulfate was started on Oct. 23. The inflammation subsided rapidly. After 3 days it was no longer possible to obtain the organisms in plate cultures. A few diplobacilli could still be found in the films. Treatment was continued until Oct. 28, when all symptoms had disappeared and diplobacilli did not develop in the plate cultures.

Cow 983.—The previous experiment was repeated. On Nov. 1, a bacteriological examination of the eyes failed to show the presence of the diplobacilli. Material obtained from beneath the membrana nictitans revealed only epithelial cells. On Nov. 1, material for inoculation was prepared by suspending three loopfuls of growth from the surface of a 24 hour agar culture in 2 cc. of bouillon. 3 drops of this suspension were instilled into the right eye. 2 days later the eye was visibly inflamed. The condition became more aggravated. 5 days after the inoculation the reddening was pronounced. The membrana nictitans was almost twice its normal size, being reddened and edematous. The lower lid was thickened. Congestion of the conjunctiva was pronounced. The characteristic yellowish white

exudate was observed. Diplobacilli were present in the smears in enormous numbers. During the observation the left eye remained normal. The diplobacilli were first observed in the plate cultures and films prepared from the membrana nictitans of the left eye 10 days after inoculation. The inflammation gradually subsided in the right eye and on Nov. 12 abnormalities could no longer be detected. On Nov. 17, the organisms were still present in material obtained from both nictitating membranes.

On the whole, the disease produced experimentally was milder than that observed in the herd. The fact that the diplobacilli constantly associated with the spontaneous disease are fully capable of reproducing the disease when introduced into the eyes of normal cattle is established by our experiments. Probably the severity of the disease may be influenced by initial injury. The organism is, however, capable of attacking the normal membranes. In our experimental inoculations precautions were always taken against injurying either the eye or the lids. It seems possible to account for the comparative mildness of the inflammations by the conditions under which the cattle were kept. The units were free from draughts. The temperature was fairly uniform and the atmosphere contained very little dust. In addition, flies were not numerous. On the other hand, the spontaneous cases varied considerably in their intensity. The incubation period was relatively a short one. It varied from 2 to 4 days in the experimental cases.

Of considerable significance is the persistence of the etiological agent in two of the three experimental cases after the disease had apparently subsided. Additional evidence was obtained when ten spontaneous cases were reexamined 3 and 4 months after the original observations. In a few cases a little discharge still persisted, but in nine cows a clinical diagnosis of ophthalmia was not justified. There was a pronounced opacity of the cornea in the other animal. From material obtained from the membrana nictitans of five cases diplobacilli were observed in the films and cultivated in plate or coagulated serum cultures.

DISCUSSION.

From the lack of literature it would seem that contagious ophthalmia is relatively rare in cattle. Practitioners in this district, however, inform us that during the early fall months eye diseases are prevalent

in certain localities. Whether these outbreaks are due to infection with the diplobacilli is not known. Allen[3] called attention to a disease of the eyes of cattle in the Province of Quebec. The symptoms manifested differed in certain respects from those observed by us. He called particular attention to a smoky or hazy appearance of the cornea with marked lacrimation and photophobia. The development of protuberances in the membrana nictitans was also recorded. Short, thick diplobacilli were encountered in small numbers in stained smears of the watery exudate. Attempts to cultivate these organisms on ordinary media failed. In one instance a culture was obtained in a serum medium. Attempts to reproduce the disease in cattle by instillation of this culture into the eyes failed.

In a translation by Kappeyney and Ward[4] of an article by Poels[5] an infectious keratitis of cattle is described. From bits of incised cornea Poels cultivated *Bacillus pyogenes*. Instillation of the culture beneath the lids produced no apparent ill effects. Injection of *Bacillus pyogenes* beneath the epithelial layer of the cornea resulted in infection.

In morphology and the ability to liquefy serum the organism isolated by Allen resembles the one obtained by us. There are, however, many clinical differences. In our cases a yellowish white, mucous or mucopurulent discharge was practically a constant accompaniment of the disease. Films from the bulk of the cases revealed the diplobacilli in large numbers. Allen calls attention to the relatively few organisms encountered in a watery exudate. In his cases the principal diagnostic sign was a smoky appearance of the eyeball. This symptom was not observed by us.

The disease did not necessarily attack neighboring cows. The cases occurred irregularly throughout the barn and finally spread to other barns. It seems possible to account for this irregular occurrence by assuming transmission by flies. They were numerous during the outbreak and were frequently observed feeding on the exudate. The disease became quiescent after the inauguration of treatment and the onset of cold weather. It is not impossible that it may

[3] Allen, J. A., *J. Am. Vet. Med. Assn.*, 1918–19, liv, 307.

[4] Kappeyney, J., and Ward, A. R., *J. Am. Vet. Med. Assn.*, 1917, li, 526.

[5] Poels, J., *Tijdschr. veeartsenijk.*, 1911, xxxviii, 758.

reappear in sporadic or epidemic form at any time, since five of the ten cows reexamined after 3 or 4 months were still carrying the diplobacilli. Since the data have been gathered together a typical but mild infection from which diplobacilli were isolated occurred in the latter part of January, 1923.

Of considerable interest is the resemblance of the diplobacillus obtained from the cows to that described by Morax and Axenfeld, Pusey,[6] and others from human conjunctivitis. The morphology appears to be the same. Certain of the growth characters of both organisms are suggestive. Both form the characteristic pits of liquefaction on the surface of coagulated serum. Both are obligatory aerobes. The addition of serum to media enhances growth. Carbohydrate fermentation has not been observed for either organism. Neither is pathogenic for laboratory animals. Of interest is the apparent specificity of zinc sulfate in the treatment of infections with the human and the bovine diplobacilli. The Morax-Axenfeld bacillus is said not to liquefy gelatin. Its failure to grow in milk is a further difference. It may be possible to explain these apparent differences in the light of more recent knowledge concerning the initial hydrogen ion concentration of culture media. The proteolytic activities of the Morax-Axenfeld bacillus in coagulated serum media suggest that under proper conditions of alkalinity digestion of gelatin and casein is possible. Both types must be compared under the same conditions before specific differences can be determined.

SUMMARY.

Twenty-four cases of an acute ophthalmia of cattle have been observed. The infection is characterized by photophobia, severe congestion of the vessels of the eyeball, conjunctivitis, congestion and edema of the membrana nictitans, edema of the eyelids, accompanied by a thick, yellowish white mucus or mucopurulent exudate. In certain cases corneal ulcers and extensive corneal opacities developed. From all cases a characteristic diplobacillus was obtained. The organism was usually observed in the exudate in large numbers. The morphology, the hemolytic properties, and the proteolytic

[6] Pusey, B., *J. Am. Med. Assn.*, 1906, xlvii, 255.

activities readily assist in its identification. Instillation of a few drops of bouillon suspensions of pure cultures beneath the eyelids of normal cattle gave rise to characteristic inflammations. The organism is not pathogenic for laboratory animals.

EXPLANATION OF PLATE 12.

FIG. 1. A spontaneous case. The lids are thickened. The characteristic exudate has accumulated at the inner canthus and has been washed down over the face.

FIG. 2. Exudate from a spontaneous case. The diplobacilli are visible in the mucous exudate. × 1,000.

FIG. 3. Diplobacilli in the mucopurulent exudate obtained from a spontaneous case. × 1,000.

FIG. 4. Colonies in a horse blood agar plate culture after 48 hours incubation. The hemolytic areas about both the surface and deep colonies are well defined. Natural size.

FIG. 5. Mucopurulent exudate obtained from Cow 897, an experimental case. × 1,000.

(Jones and Little: Infectious ophthalmia of cattle.)

STUDIES ON PNEUMOCOCCUS IMMUNITY.

II. Active Immunization of Monkeys against Pneumococcus Types II, III, and IV Pneumonia with the Homologous Pneumococcus Vaccine.*

By RUSSELL L. CECIL, M.D., and GUSTAV I. STEFFEN.

(*From the Hygienic Laboratory of the United States Public Health Service and the Second Medical (Cornell) Division and Pathological Laboratory of Bellevue Hospital, New York.*)

(Received for publication, March 28, 1923.)

In a previous communication (1) it has been shown that the subcutaneous or intravenous injection of monkeys with Pneumococcus Type I vaccine confers on them a complete immunity against experimental Pneumococcus Type I pneumonia. In this article the results of similar experiments with Pneumococcus Types II, III, and IV will be reported. Two species of monkeys have been employed in these experiments, the Philippine *Macacus syrichtus* and the South American *Cebus capucinus*. Both species are quite susceptible to pneumococcus and when inoculated intratracheally with a virulent culture practically always develop pneumonia.

Methods.

Ordinary saline pneumococcus vaccine was used for the experiments. The method of preparation has been described in Paper I of this series (1). In all instances the monkeys received three subcutaneous injections of vaccine at intervals of 5 to 7 days, the total dosage varying from 120 to 200 billion pneumococci. The first dose was usually small, the second and third much larger. As in the previous experiments, the injections of vaccine were made in the back or abdomen and excited only a mild grade of local reaction.

In testing the immunity of the vaccinated monkeys, the same pneumococcus strains were used for the intratracheal injections of living culture that had been employed in making the vaccines. These tests were carried out 2 or 3 weeks after the completion of vaccination. The technique of intratracheal inoculation

* This study received financial aid from the Influenza Fund of the Metropolitan Life Insurance Company, New York.

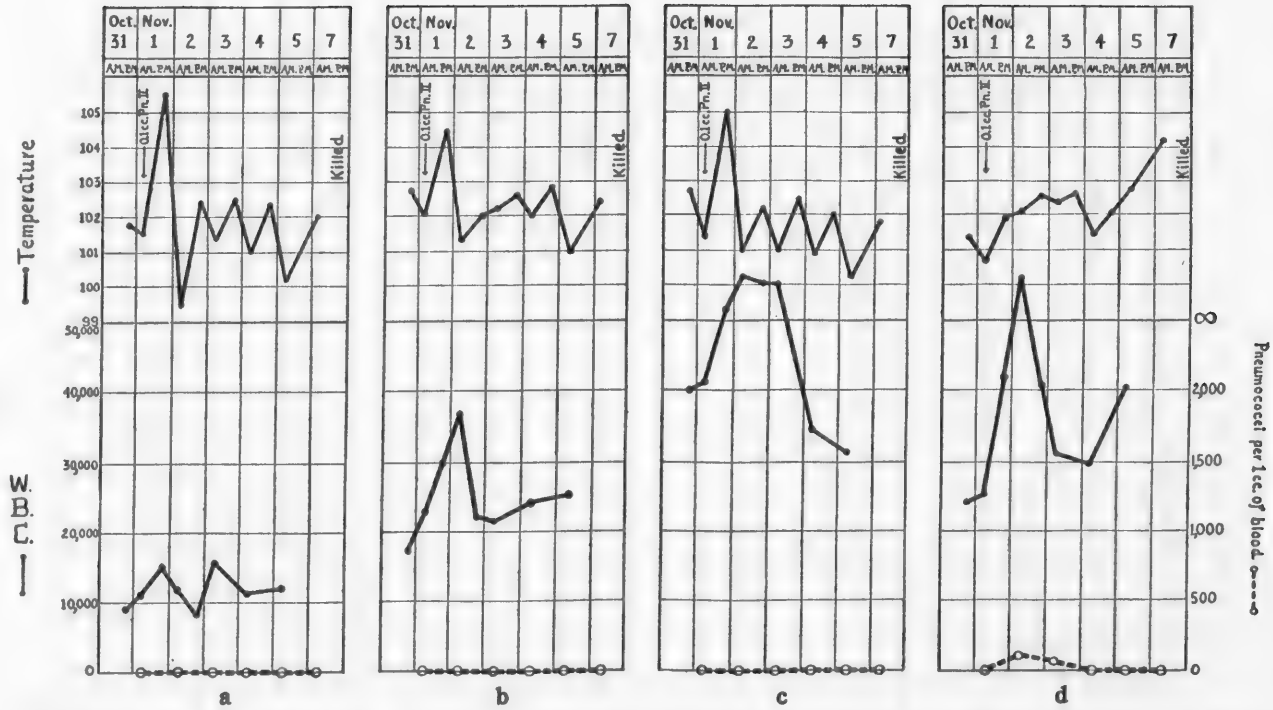
has also been previously described. The dose of culture injected in these experiments varied from 0.1 to 0.000001 cc. for each monkey, depending on the virulence of the strain. In every experiment an unvaccinated control was inoculated simultaneously with the vaccinated monkeys, and in every case the control came down promptly with pneumonia. Following the inoculations each monkey was examined daily, usually for 7 or 8 days. The rectal temperature was taken twice daily and leucocyte counts and blood cultures were made once a day. The monkeys that did not die following inoculation with cultures were killed at the conclusion of the experiment. Complete autopsies were performed on all animals and microscopic sections from the lungs were studied. Routine postmortem cultures were taken from the lungs and heart's blood.

Active Immunity against Pneumococcus Type II Pneumonia Following Three Subcutaneous Injections of Pneumococcus Type II Vaccine.

The first experiment in this study was carried out with Pneumococcus Type II. A virulent culture, fatal for mice in doses of 0.00000001 cc. of broth culture, was used for the preparation of the vaccine and also for the subsequent immunity tests.

Experiment 1.—Three Philippine monkeys were vaccinated subcutaneously as follows: Sept. 3, 1921. No. 1 (1,654 gm.), No. 2 (1,395 gm.), and No. 3 (2,167 gm.) received each 10 billion (1 cc.) of Pneumococcus Type II vaccine subcutaneously. Sept. 10. Each monkey received 80 billion (2 cc.) of Pneumococcus Type II vaccine subcutaneously. Sept. 17. Each monkey received 100 billion (2 cc.) of Pneumococcus Type II vaccine subcutaneously. There was no general reaction noticeable after any of the injections. Monkeys 1 and 2 each developed a small hard nodule at the site of the third inoculation. These nodules disappeared after about 1 week. Oct. 1 (2 weeks after the third injection of vaccine). The three vaccinated monkeys and a control monkey, No. 4 (2,412 gm.), each received intratracheally 0.1 cc. of a broth culture of virulent Pneumococcus Type II.

The results of this experiment are shown in Text-fig. 1. The vaccinated monkeys reacted with a sudden rise in temperature, which reached its maximum about 6 hours after inoculation. The next morning the temperatures of all three vaccinated monkeys were normal and remained so. In each instance this rise of temperature was accompanied by a temporary increase in the number of leucocytes. The monkeys were healthy and lively throughout the experiment and their blood cultures remained sterile. The control monkey became ill shortly after inoculation with the Pneumococcus Type II culture and ran a typical course of lobar pneumonia with marked leucocytosis and pneumococcemia. All four monkeys were killed on the 7th day after the infecting dose was given. At autopsy none of the vaccinated monkeys showed any evidence of consolidation. In two



TEXT-FIG. 1, *a* to *d*. Active immunity against Pneumococcus Type II pneumonia following vaccination with Pneumococcus Type II vaccine. (*a*), (*b*), and (*c*) Monkeys 1, 2, and 3; each received 190 billion Pneumococcus Type II vaccine subcutaneously. (*d*) Monkey 4; control.

of the vaccinated animals a few small petechial hemorrhages were found in the lungs, but microscopic sections from the foci revealed only red blood corpuscles in the alveoli, with no exudation of leucocytes. Cultures from the hemorrhagic areas were sterile. The control monkey showed complete consolidation of the right lower lobe, with typical pneumonic exudate from which Pneumococcus Type II was isolated in pure culture.

Protection Tests.—Blood was taken from the three vaccinated monkeys just before their active immunity was tested to determine the protective power of their serums in mice. No protective action could be demonstrated against Pneumococcus Type II in any of the serums, even when doses as minute as 0.0000001 cc. of culture were used.

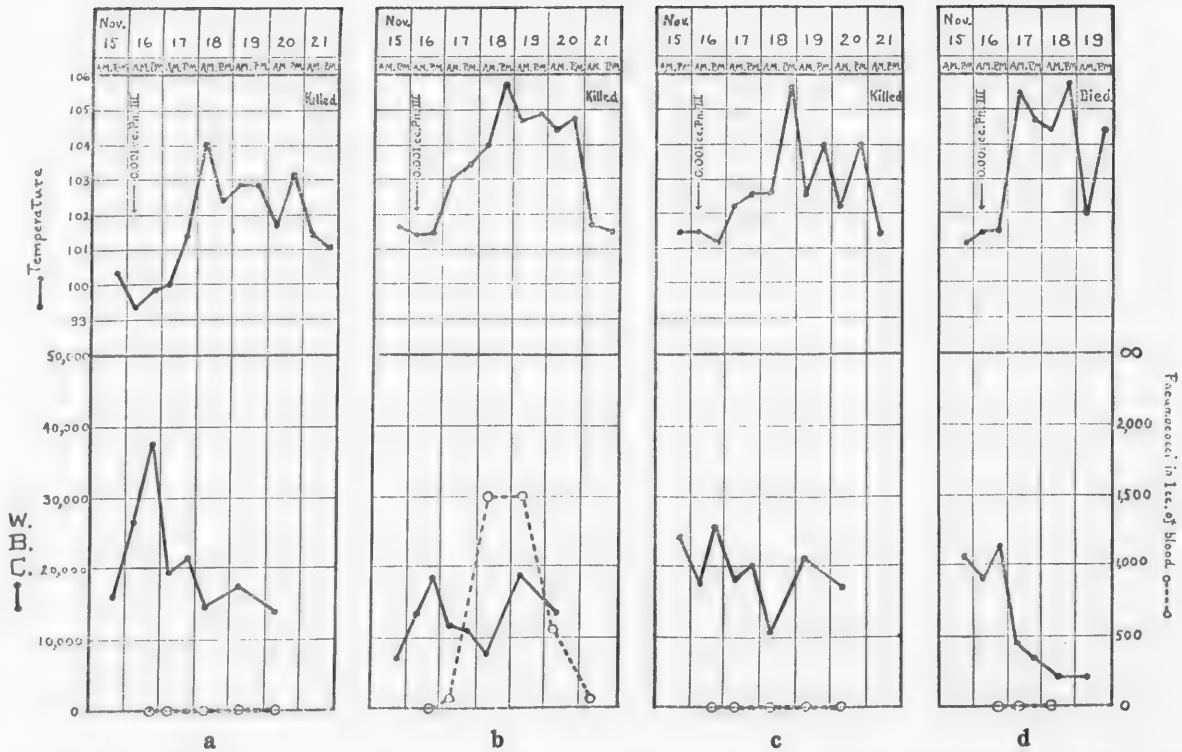
From this experiment it may be inferred that three subcutaneous injections of Pneumococcus Type II vaccine afford complete protection to monkeys against Pneumococcus Type II pneumonia.

Active Immunity against Pneumococcus Type III Pneumonia Following Three Subcutaneous Injections of Pneumococcus Type III Vaccine.

The next problem was to determine the effectiveness of prophylactic vaccination against Pneumococcus Type III pneumonia. The technique in the Type III experiments was similar in every respect to that employed in the Pneumococcus Type II experiments.

Experiment 2.—Oct. 18, 1921. Three *Cebus capucinus* monkeys, No. 5 (1,500 gm.), No. 6 (1,250 gm.), and No. 7 (1,432 gm.), received each 20 billion (0.5 cc.) Pneumococcus Type III vaccine subcutaneously. Oct. 25. Each monkey received 40 billion (1 cc.) Pneumococcus Type III vaccine subcutaneously. Nov. 1. Each monkey received 60 billion (1.5 cc.) Pneumococcus Type III vaccine subcutaneously. The monkeys developed small firm nodules at the sites of these injections, but no other reaction. Nov. 15 (2 weeks after the last injection of vaccine). Each of the three vaccinated monkeys received 0.001 cc. of an 18 hour broth culture of Pneumococcus Type III (Hughes) intratracheally. An unvaccinated control monkey, No. 8 (1,450 gm.), received the same amount of culture intratracheally.

The results of the experiment are shown in Text-fig. 2. All four monkeys showed a rise of temperature following the inoculation with Pneumococcus Type III culture, but in the vaccinated monkeys the temperature returned to normal, on the 5th or 6th day after inoculation, whereas the control monkey ran a high fever until death occurred on the 4th day. The vaccinated monkeys showed a moderate leucocytosis following the intratracheal inoculation. In the control monkey a marked leucopenia developed almost at once. One of the vaccinated monkeys, No. 6, had pneumococci in the blood; in the other two the blood cul-



TEXT-FIG. 2, *a* to *d*. Active immunity against Pneumococcus Type III pneumonia following vaccination with Pneumococcus Type III vaccine. (*a*), (*b*), and (*c*) Monkeys 5, 6, and 7; each received 120 billion Pneumococcus Type III vaccine subcutaneously. (*d*) Monkey 8; control.

tures remained sterile. Strangely enough, no pneumococci were found in the blood of the control monkey. The most significant thing in this experiment was the difference in appearance between the vaccinated monkeys and the control. The former remained lively and well during the entire period of observation. The control became very sick at once and died on the 4th day.

The vaccinated monkeys were killed on the 6th day. Autopsies showed normal lungs in Monkeys 5 and 7. Several small patches of interstitial pneumonia were found in the right lower lobe of No. 6. The control monkey had a frank consolidation of the right middle and lower lobes. Postmortem cultures were negative in Monkeys 5 and 7. Cultures from the right lower lobe of No. 6 yielded Pneumococcus Type III. Cultures from the control were overrun with *B. bronchisepticus*, but a few Gram-positive diplococci were seen in microscopic sections stained for bacteria.

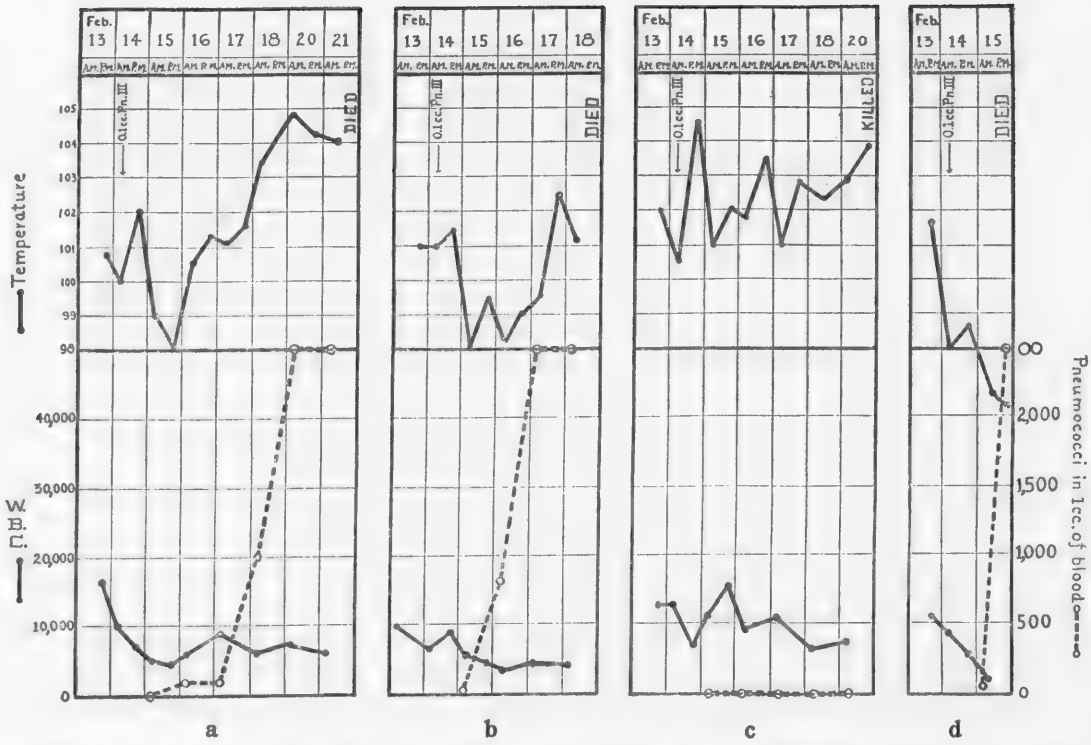
Protection Tests.—Blood from the three vaccinated monkeys showed no protective bodies against Pneumococcus Type III in mice.

In this experiment complete protection against Pneumococcus Type III was obtained in two of the vaccinated monkeys, partial protection in the third. It is hard to explain why these vaccinated monkeys, though immune to pneumonia, sometimes show a temporary rise in temperature following inoculation with the living culture. In some instances an acute bronchitis is excited which, however, does not develop into pneumonia. In others the fever may be the expression of a foreign protein reaction.

A second experiment was carried out with Pneumococcus Type III vaccine in the hope of obtaining complete protection in all the vaccinated monkeys. In this experiment larger doses of vaccine were employed.

Experiment 3.—Jan. 23, 1922. Three *Cebus capucinus* monkeys, No. 9 (1,328 gm.), No. 10 (1,360 gm.), and No. 11 (1,244 gm.), received each 1 billion (1 cc.) Pneumococcus Type III vaccine (Hughes) subcutaneously. Jan. 28. Each monkey received 80 billion (1 cc.) Pneumococcus Type III vaccine subcutaneously. Feb. 1. Each monkey received 120 billion Pneumococcus Type III vaccine subcutaneously. Feb. 13. No. 9 has developed a small ulcer at the site of the last inoculation. The other monkeys show only the usual small nodules. Feb. 14 (2 weeks after the third injection of vaccine). Each monkey received 0.1 cc. of an 18 hour broth culture of Pneumococcus Type III (Hughes) intratracheally. An unvaccinated monkey, No. 12 (1,840 gm.), received the same amount (0.1 cc.) of culture intratracheally.

The results are shown in Text-fig. 3. Monkey 11 remained lively and well. There was a temporary rise in temperature, but the leucocytes were normal and



TEXT-FIG. 3, *a* to *d*. Active immunity against Pneumococcus Type III pneumonia following vaccination with Pneumococcus Type III vaccine. (*a*), (*b*), and (*c*) Monkeys 9, 10, and 11; each received 200 billion Pneumococcus Type III vaccine subcutaneously. (*d*) Monkey 12; control.

the blood culture remained sterile. The other two vaccinated monkeys became ill, developed a leucopenia and heavy pneumococcemia and died, one on the 5th and the other on the 7th day of the disease. The control monkey died the day after inoculation with an overwhelming pneumococcemia.

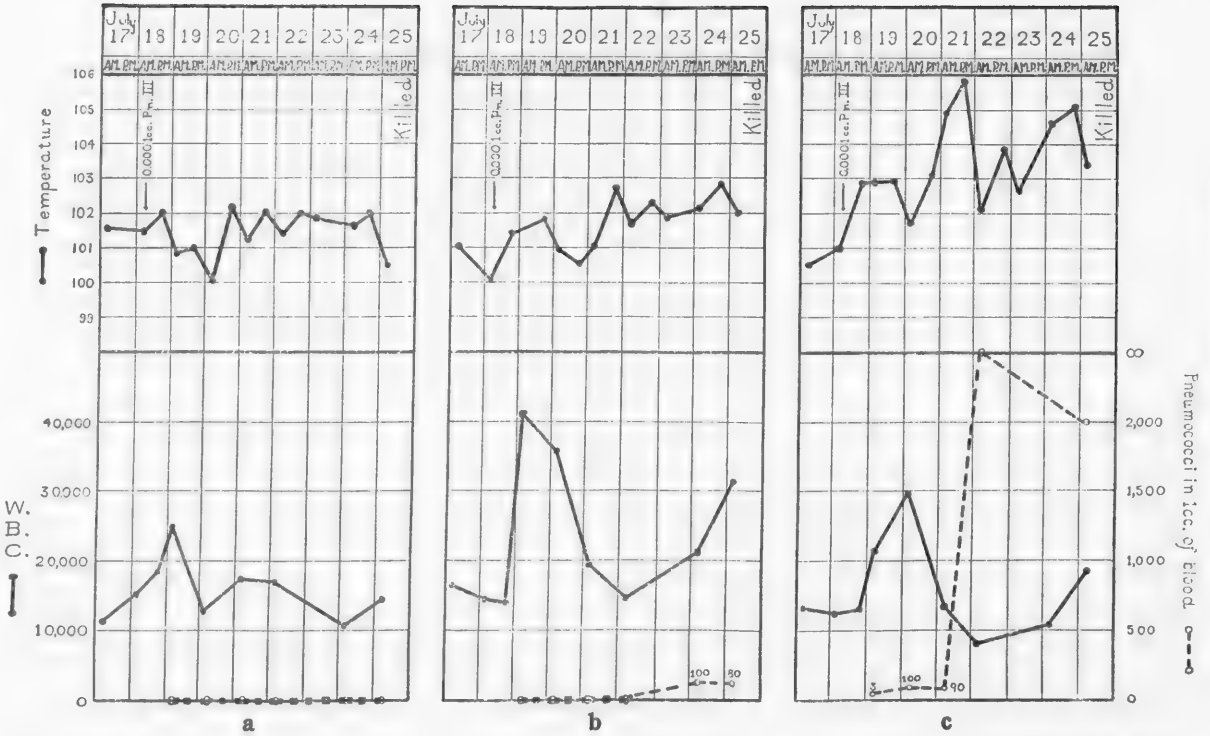
Autopsies were performed on all four monkeys. Monkey 11 showed a few petechial hemorrhages in the lungs, but no consolidation. Monkeys 9 and 10 both had lobar pneumonia, the former in the right lower lobe, the latter in both the right and left lower lobes. The control monkey, No. 12, showed an early lobar pneumonia in the right lower lobe and several partly healed foci of pulmonary tuberculosis. Postmortem cultures from the lungs and heart's blood of Monkeys 9, 10, and 12 yielded Pneumococcus Type III. Cultures from Monkey 11 were negative, except for a few colonies of Pneumococcus Type III which were found on cultures from the trachea.

In this experiment only one of the three vaccinated monkeys resisted infection with Pneumococcus Type III. The other two developed a Pneumococcus Type III pneumonia. A careful study of the protocols indicates that too large an infecting dose was used in this experiment. This is shown by the fulminating course which the infection pursued in the control. As it was, the two vaccinated monkeys that developed pneumonia lived several days longer than the unvaccinated monkey.

One more Type III experiment was undertaken, this time with another strain of pneumococcus.

Experiment 4.—June 16, 1922. Two *Cebus capucinus* monkeys, No. 13 (1,200 gm.) and No. 14 (1,550 gm.), received each 12 billion (1 cc.) Pneumococcus Type III (Bowen) vaccine subcutaneously. June 23. Each monkey received 50 billion (2 cc.) Pneumococcus Type III vaccine subcutaneously. June 30. Each monkey received 138 billion (2 cc.) Pneumococcus Type III vaccine subcutaneously. July 18 (2½ weeks after the last injection of vaccine). The two vaccinated monkeys and a control monkey, No. 15 (2,600 gm.), each received 0.001 cc. of an 18 hour broth culture of Pneumococcus Type III (Bowen) intratracheally.

Monkey 13 remained lively and well, but Monkey 14 became moderately sick. The control monkey, No. 15, became quite sick, and probably would have died had it not been killed on the 8th day. Text-fig. 4 shows the temperature, leucocyte, and blood culture curves. Monkey 13 showed no changes in temperature or leucocyte count, and the blood remained sterile. Monkey 14 developed no fever, but the leucocytes rose, and pneumococci appeared in the blood in moderate numbers on the 7th day of observation. The control monkey ran a high fever, the leucocytes rose to 29,600, and a heavy pneumococcemia appeared on the 4th day following inoculation.



TEXT-FIG. 4, *a* to *c*. Active immunity against Pneumococcus Type III pneumonia following vaccination with Pneumococcus Type III vaccine. (*a*) and (*b*) Monkeys 13 and 14; each received 200 billion Pneumococcus Type III vaccine subcutaneously. (*c*) Monkey 15; control.

All three monkeys were killed on the 8th day. No. 13 showed normal lungs; No. 14 presented several small patches of consolidation in both lower lobes. The control, No. 15, showed an interstitial pneumonia of the right lower lobe. Post-mortem cultures from the lungs and heart's blood of Monkey 13 were sterile. Monkey 14 and the control both yielded Pneumococcus Type III from the heart's blood and also from the consolidated areas in the lungs.

Protection Tests.—Protection tests with blood from the two vaccinated monkeys were carried out on mice, but no protective substance could be demonstrated in either monkey.

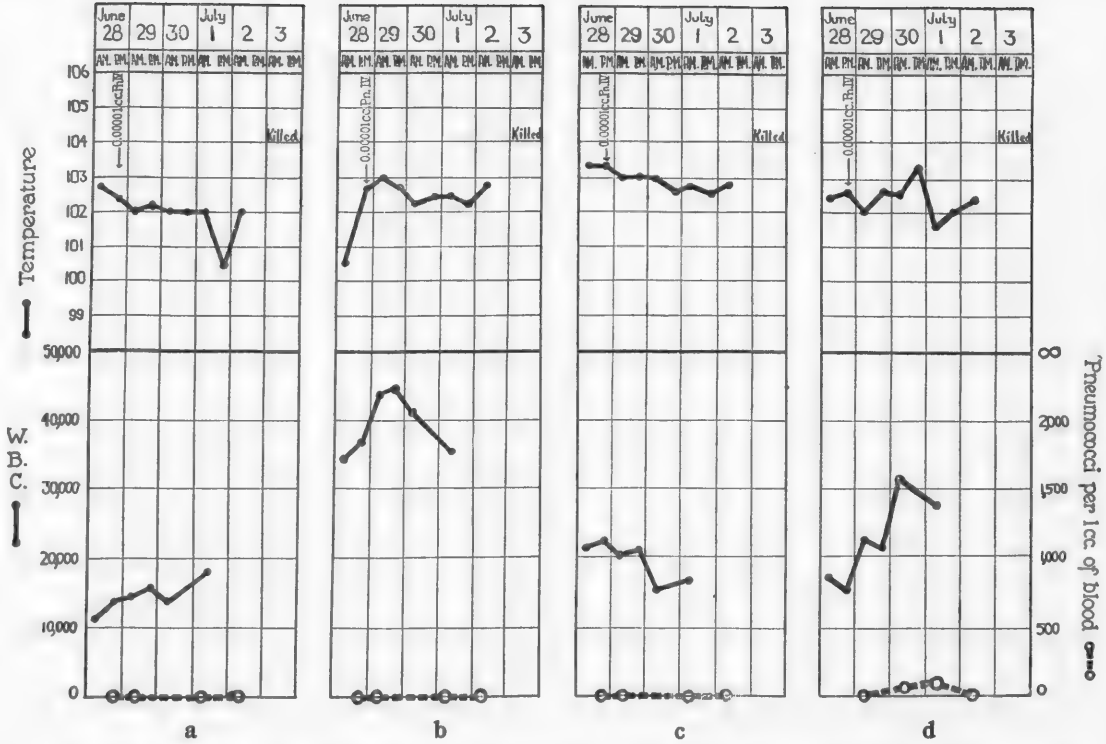
This experiment, like the two preceding ones with Pneumococcus Type III, was not entirely successful in that one of the vaccinated monkeys was not sufficiently immune to resist infection. In view of the difficulties experienced by previous workers in immunizing animals against Pneumococcus Type III, we had expected to meet with less success with Type III than with the other types. Altogether, eight monkeys were vaccinated against Pneumococcus Type III. Four of them resisted infection and four developed Type III pneumonia.

Active Immunity against Pneumococcus Type IV Pneumonia Following Three Subcutaneous Injections of Pneumococcus Type IV Vaccine.

Pneumococcus Type IV is really not a type but a heterogeneous group of pneumococci that do not fall into any one of the three fixed types. The so called Type IV strains rarely cross-agglutinate or cross-protect, but it seemed desirable to carry out one Type IV experiment on monkeys, using the same strain of pneumococcus for vaccine and for testing the immunity.

Experiment 5.—May 29, 1921. Three *Macacus syrichtus* monkeys, No. 16 (1,775 gm.), No. 17 (1,430 gm.), and No. 18 (1,410 gm.), received each 20 billion (0.5 cc.) Pneumococcus Type IV vaccine subcutaneously. June 4. Each monkey received 40 billion (1 cc.) Pneumococcus Type IV vaccine subcutaneously. June 11. Each monkey received 60 billion (1 cc.) Pneumococcus Type IV vaccine subcutaneously. June 28 (2½ weeks after the last injection of vaccine). Each monkey received 0.00001 cc. of an 18 hour broth culture of Pneumococcus Type IV intratracheally. At the same time, an unvaccinated control, No. 19 (2,300 gm.), received the same amount of culture intratracheally.

The results are shown in Text-fig. 5. The three vaccinated monkeys remained perfectly well. There was no noteworthy rise in the temperature or leucocyte count, and the blood cultures remained sterile. The control monkey showed a slight rise in temperature, the leucocytes jumped from 17,000 to 32,000 and the



TEXT-FIG. 5, *a* to *d*. Active immunity against Pneumococcus Type IV pneumonia following vaccination with Pneumococcus Type IV vaccine. (*a*), (*b*), and (*c*) Monkeys 16, 17, and 18; each received 120 billion Pneumococcus Type IV vaccine subcutaneously. (*d*) Monkey 19; control.

blood cultures yielded Pneumococcus Type IV on the 3rd and 4th days of the disease. During this period the control was very quiet but did not appear acutely ill.

On the 6th day all four monkeys were killed. The three vaccinated monkeys were found to have normal lungs. The control monkey showed small patches of interstitial pneumonia in the right lower and the left upper lobes. Postmortem cultures from the lungs of the vaccinated monkeys were sterile. Cultures from the patches in the lungs of the control yielded Pneumococcus Type IV.

Protection Tests.—Serum was collected from the three immunized monkeys just before the immunity test was made, and protection tests against the homologous strain of Pneumococcus Type IV were carried out on mice. No protective bodies could be demonstrated in any of the three serums.

This experiment would have been more convincing if the control had shown a more extensive pneumonia. However, pneumonia was present in the control and absent in the three vaccinated monkeys,—evidence enough to show that active immunity could be established against Pneumococcus Type IV as well as against the three fixed types.

DISCUSSION.

It was reasonable to suppose that if monkeys could be immunized against Pneumococcus Type I, they could also be immunized against the other three types, for the pneumococci all belong to the same biological species and have almost identical morphological and cultural characteristics. It is only in respect to immunological traits that they vary among themselves.

It is more difficult to obtain a high immunity against Pneumococcus Type III than it is against the other types. It is possible that the thick capsule which characterizes this type may interfere in some way with the release of the antigen and the consequent stimulation of the mechanism which produces the immune substance. The same factor that prevents the development of a high active immunity may also be responsible for the high death rate in Type III pneumonia in man. In the one instance, killed pneumococci, in the other, living pneumococci, fail to stimulate a sufficiently sharp response by the immune mechanism.

Monkeys tolerate large doses of pneumococcus vaccine remarkably well. In the present study the local reactions were insignificant and constitutional reactions were slight or entirely absent.

In the experiments with Pneumococcus Type I, previously reported, three subcutaneous injections of monkeys with Pneumococcus Type I vaccine often stimulated the production of specific protective substance in the blood of the vaccinated monkeys. In a similar way, protective bodies could be demonstrated after three intravenous injecjections of Type I vaccine. In the experiments with Pneumococcus Types II, III, and IV, three subcutaneous injections of vaccine failed in every instance to produce protective substance in the blood, even when the monkey was immune to infection. Evidently it is more difficult to stimulate protective bodies against Types II, III, and IV than against Type I. Judging from experience with other animals, however, protective bodies against all types could be developed in monkeys if repeated injections of vaccine were administered either subcutaneously or intravenously.

CONCLUSIONS.

1. Three subcutaneous injections of Pneumococcus Type II vaccine confer on monkeys a complete immunity against experimental Pneumococcus Type II pneumonia. A similar protection can be bestowed on monkeys against Pneumonococcus Type IV pneumonia by three subcutaneous injections of a vaccine prepared from the same strain of pneumococcus.

2. The subcutaneous injection of monkeys with three doses of Pneumococcus Type III vaccine confers a complete immunity against this type in only 50 per cent of cases (four out of eight monkeys vaccinated).

3. In spite of the immunity induced in monkeys by three subcutaneous injections of Pneumococcus Types II, III, and IV vaccine, specific protective bodies against the homologous types are not demonstrable in their serums when the vaccine is so administered.

BIBLIOGRAPHY.

1. **Cecil, R. L., and Steffen,** G. I., *J. Exp. Med.*, 1921, xxxiv, 245.

STUDIES ON PNEUMOCOCCUS IMMUNITY.

III. The Nature of Pneumococcus Antigen.*†

By WILLIAM A. PERLZWEIG, Ph.D., and GUSTAV I. STEFFEN.

(From the Hygienic Laboratory of the United States Public Health Service and the Second Medical (Cornell) Division and Pathological Department of Bellevue Hospital, New York.)

(Received for publication, March 28, 1923.)

In the two preceding articles of this series it has been shown by Cecil and Steffen (1) that monkeys may be immunized against pneumococcus pneumonia by subcutaneous or intravenous injections of pneumococcus vaccine. Inasmuch as rather large doses of vaccine were required to obtain complete immunity by subcutaneous injections it seemed desirable to investigate the chemical nature of pneumococcus antigen with the hope that some method might be devised for separating the immunizing fraction from the toxic fraction of the microorganism and thereby provide a more practical agent for prophylactic vaccination against pneumonia in man.

Methods.

Most of the antigens tested in this study were prepared from a highly virulent strain of Pneumococcus Type I. Later on, in order to corroborate the results obtained with Pneumococcus Type I, some experiments were also carried out with Pneumococcus Types II and III.

The experiments were all performed on white mice. The vaccines and antigens were in every instance administered subcutaneously in a volume of 0.5 cc. When two injections of vaccine, or antigen, were administered, an interval of 6 or 7 days elapsed between the two injections. When three or four injections of antigen were given the interval between the injections was 3 or 4 days. The immunity

* This work also received financial aid from the Influenza Fund of the Metropolitan Life Insurance Company, New York.

† Preliminary reports on this investigation were presented at the New York meeting of the American Chemical Society on September 9, 1921, and before the New York Pathological Society in November, 1921 (Perlzweig, W. A., *Proc. New York Path. Soc.*, 1921, xxi, N. S., 133).

of the vaccinated mice was usually tested 9 to 10 days after the last injection of antigen by inoculating them intraperitoneally with various dilutions of a broth culture of the homologous type of pneumococcus. The culture was diluted with plain broth and the volume injected was invariably 0.5 cc. The cultures were carefully controlled by examination of stained smears and agglutination and bile solubility tests. Each experiment was controlled by injecting unvaccinated mice intraperitoneally with one or more lethal doses of pneumococcus culture (0.0000001 and 0.00000001 cc.). All of these control mice, a total of 138, without a single exception, died in less than 72 hours and pneumococci were found in the peritoneal exudate of each of them. In view of the invariably high virulence of the strains employed each survival among the experimental mice must be regarded as significant and showing the production of definite immunity by the antigen in question. A mouse was counted a survival if it lived for 5 days or more after the inoculation of the virulent culture. Usually the mice remained under observation for 7 to 10 days after the inoculation. Each dead mouse was dissected and smears from the peritoneal exudate were examined for pneumococci. In cases of doubt cultures were made on blood agar plates.

I. Immunization with a Saline Suspension of Killed Pneumococcus Type I.

It was first established that small doses of a saline suspension of Pneumococcus Type I, killed by heating at 60°C. for 1 hour, induce immunity in mice against subsequent infection. The importance of giving a small injection of vaccine, followed 1 week later by a larger dose, is demonstrated in the following two experiments.

Experiment 1.—Nine mice received each a single subcutaneous injection of 700 million (0.5 cc.) Pneumococcus Type I saline vaccine. 10 days later they were injected intraperitoneally with a virulent culture of Pneumococcus Type I.

No. of mice.	Culture.	Died.	Survived.
	cc.		
3	0.000001	1	2
3	0.00001	1	2
3	0.0001	3	0

Experiment 2.—Sixteen mice were each injected with 100 million (0.5 cc.) Pneumococcus Type I saline vaccine. 7 days later they received a second dose of 700 million each. 10 days after the last injection of vaccine they were all inoculated intraperitoneally with a broth culture of virulent Pneumococcus Type I.

No. of mice.	Culture.	Died.	Survived.
	cc.		
2	0.000001	0	2
3	0.00001	0	3
3	0.0001	0	3
3	0.001	0	3
3	0.01	0	3
2	0.1	1	1

A moderate immunity was induced by one subcutaneous injection of vaccine (Experiment 1), but a much higher degree of immunity was obtained when two injections were given.

Wadsworth (2) found recently in studying the same question that it took no less than six injections of 6 billion each of killed pneumococci to protect white mice against an infecting dose of 0.001 cc. of a culture of Pneumococcus Type I with about the same virulence as that of the strain used in these experiments. This divergence between the observations of Wadsworth and our own afford some support to the contention of Much (3) that very small initial doses of vaccine are essential to the development of a high degree of immunity.

II. Immunization with the Protein Fraction of the Pneumococcus.

The immunizing properties of the protein fraction of the pneumococcus were studied next. In order to alter the chemical nature of the proteins as little as possible the following methods of separation were used.

(*a*) *Rowland's Method* (*4*).—Moist sediments of living pneumococci were thoroughly mixed with twice their weight of anhydrous sodium sulfate and alternately frozen and thawed at 37°C. for several days. Sufficient water was added to make a saturated solution of sodium sulfate at 37°C. The precipitated protein was washed with additional sodium sulfate solution, redissolved in very dilute (0.01 N) sodium carbonate solution, and freed of the adhering salt by dialysis. As in Rowland's study of the plague bacillus the resulting product was found to be chiefly nucleoprotein as shown by the presence of organic phosphorus and of the purine bases, guanine and adenine, on hydrolysis with nitric acid. The slightly alkaline solution was filtered

through a diatomaceous earth filter (Mandler), tested for sterility, and inoculated into mice to determine its antigenic value. In vaccinating mice with the various fractions and extracts of pneumococcus the dosage was determined by the concentration of the bacterial suspension from which the fraction had been derived; for example, if the original suspension contained 100 billion pneumococci per cc., mice which received 0.1 cc. of the protein fraction were recorded as having received 10 billion pneumococci.

Experiment 3.—Eleven mice each received two subcutaneous doses of an alkaline solution of Pneumococcus Type I protein, corresponding to 200 million and 4 billion pneumococci respectively. 10 days after the second dose, which was given 7 days after the first, the mice were injected intraperitoneally with a broth culture of virulent Pneumococcus Type I.

No. of mice.	Culture.	Died.	Survived.
	cc.		
4	0.0001	0	4
3	0.001	1	2
4	0.01	1	3

(*b*) *Solution of Pneumococci in Bile Salts.*—The property of pneumococci to dissolve in bile and in solutions of bile salts was utilized for the further study of pneumococcus antigen. To saline suspensions of Pneumococcus Type I sodium choleate was added to make a 1 per cent solution, also 0.2 per cent disodium phosphate, and the mixture incubated at 37°C. for 2 to 4 hours, until solution of the pneumococci was complete or nearly so. The undissolved material was separated by centrifugalization and the supernatant fluid thrown into nine volumes of 95 per cent alcohol. The abundant white precipitate was allowed to settle over night, filtered off, and washed free of the bile salts with 90 per cent alcohol. The same nucleoprotein characteristics were found in this substance as in that obtained by Rowland's method. It is soluble in water and in slightly alkaline solutions, coagulated by boiling in the presence of a little acid, contains organic phosphorus and purine bases, and responds to all protein color reactions. A slightly alkaline filtered solution was used in the following experiment.

Experiment 4.—Nine mice were each injected subcutaneously with a protein solution obtained from a bile salt solution of Pneumococcus Type I, the dose corresponding to 2 billion pneumococci. 10 days later the mice were inoculated intraperitoneally with a broth culture of virulent Pneumococcus Type I.

No. of mice.	Culture.	Died.	Survived.
	cc.		
3	0.00001	1	2
3	0.0001	2	1
3	0.001	2	1

From these experiments it will be seen that the antigen is either a part of the protein fraction of the pneumococcus or else is some substance attached to it.

III. Immunization with Proteolytic and Autolytic Cleavage Products.

Rosenow (5) claims to have shown that autolyzed pneumococci contained an antigen. Rowland (4) showed that his plague bacillus nucleoprotein lost its toxic properties and none of its immunizing properties if permitted to autolyze for as long as 40 days. Jobling and Strouse (6) showed that pneumococcus extracts obtained by digestion with leucoprotease raised the opsonic index in rabbits considerably. Hirschfelder (7) digested cultures of pneumococci with pancreatin for 15 minutes and was able to confer active immunity upon rabbits by injecting the acid filtrates from these digests. So far as the writers are aware Hirschfelder has not reported any further studies along this line since 1912. The above indications that the antigen appears to be resistant to proteolytic ferments raises the time-honored question: Is antigen necessarily of protein nature? It will be remembered that Jacoby's "protein-free" ricin was shown by Osborne, Mendel, and Harris (8) to be albumin-like in nature, when they demonstrated its digestibility with trypsin and consequent loss of antigenic property. In the following experiments it was established that prolonged digestion with trypsin does not destroy the pneumococcus antigen. Protein fractions of pneumococci prepared as described above either by bile salt solution and precipitation with alcohol or by Rowland's sulfate method were digested with trypsin in an alkaline medium, pH 7.7 to 8.0, at 37°C. from 4 to 21 days in

the presence of chloroform. The latter was used for the double purpose of preserving the digest and of removing the effects of the lipoidal antiferments (Jobling and Strouse (6)).[1] The progress of digestion was followed by determinations of non-protein nitrogen by Greenwald's trichloroacetic acid method of precipitation (9) and Folin's micro Kjeldahl method (10). It was found that alkaline or acid filtrates of such digests in which the non-protein nitrogen reached 85 per cent of the total nitrogen retained the original immunizing effect on white mice.

Experiment 5.—A solution of Pneumococcus Type I protein, obtained by dissolving pneumococci in sodium choleate, precipitating with alcohol, and redissolving in distilled water, was digested with trypsin for 4 days and gave a $\frac{\text{Non-protein nitrogen}}{\text{Total nitrogen}}$ ratio of 57 per cent. The total nitrogen in the filtrate was 25.2 mg. per 100 cc., of which 1 cc. represented 40 billion pneumococci. One dose, corresponding to 1 billion pneumococci, was administered subcutaneously to each of six mice, which were inoculated intraperitoneally 10 days later with a virulent Pneumococcus Type I culture.

No. of mice.	Culture.	Died.	Survived.
	cc.		
3	0.000001	2	1
3	0.00001	1	2

Experiment 6.—In this experiment the same solution as above was used but in this instance it was digested for 25 days. The $\frac{\text{Non-protein nitrogen}}{\text{Total nitrogen}}$ ratio in the filtrate was 80 per cent. Six mice were each injected subcutaneously with the equivalent of 2 billion pneumococci. 10 days later they were inoculated intraperitoneally with a culture of virulent Pneumococcus Type I.

No. of mice.	Culture.	Died.	Survived.
	cc.		
3	0.000001	1	2
3	0.00001	1	2

In another experiment a Pneumococcus Type I protein, obtained by Rowland's method, was used. The digestion with trypsin was in this instance continued for 21 days.

[1] Jobling and Strouse (6), p. 862.

Experiment 7.—Ten mice were injected subcutaneously with two doses of this preparation, equivalent to 200 million and 4,000 million respectively of killed pneumococci. The interval between the injections was 7 days. 11 days after the last injection of antigen the mice were inoculated intraperitoneally with a culture of virulent Pneumococcus Type I.

No. of mice.	Culture.	Died.	Survived.
	cc.		
10	0.01	2	8

Autolytic digests of pneumococci were also tested in the same way, with similar results. But the digestion of bacterial protein by autolysis is so much slower than that by proteolytic enzymes that the process cannot be considered advantageous.

Hirschfelder's method of obtaining bacterial antigens was carried out with some modifications. Instead of using cultures for digestion with trypsin, saline suspensions of freshly centrifugalized pneumococci were used. The suspensions were made alkaline with sodium carbonate and sodium phosphate to the reaction of pH 7.6 to 8.0. 0.2 per cent of a 1: 1 mixture of trypsin (purified, Parke, Davis and Company) and holadin, an extract of the pancreatic enzymes (Fairchild Brothers and Foster), was added. Holadin was used because of its good lipolytic properties, with the object of lessening the possible antitryptic effects of the bacterial fats and lipoids. Chloroform was also added for the same reason and in addition to act as a preservative. The digestion was permitted to continue for 18 hours at 37°C., the undigested portion was separated by centrifugalization, and the supernatant fluid was carefully acidified with dilute hydrochloric acid until the maximum precipitation of the alkali metaproteins was effected. The precipitate was permitted to settle for several hours, removed by centrifugalization, and the supernatant fluid filtered through a Mandler filter. The resulting filtrate contained only 25 per cent of the original nitrogen of the bacterial suspension, all of which was in the non-coagulable state. Precipitation and color reactions showed that amino-acids, polypeptides, and proteoses were present in this filtrate. The presence of proteoses was shown by precipitation tests with trichloroacetic and picric acids. The presence of amino-acids was ascertained by a positive ninhydrin reaction.

The biuret, Millon, and xanthoproteic reactions were likewise positive. The biuret test was of the pink-violet color, indicative chiefly of the lower peptones and polypeptides. It was possible to obtain with this filtrate a high grade of immunity in mice against Pneumococcus Type I.

Experiment 8.—In this experiment the mice received two subcutaneous doses of antigen 7 days apart. 10 days after the second injection the mice were inoculated intraperitoneally with a virulent Pneumococcus Type I culture as in preceding experiments.

Dose of antigen in millions of pneumococci.	Culture.	No. of mice.	Died.	Survived.
	cc.			
140 + 700	0.00001	4	3	1
140 + 1,400	0.00001	6	2	4

In the next experiment larger doses of antigen were employed with excellent results.

Experiment 9.—

Dose of antigen in millions of pneumococci.	Culture.	No. of mice.	Died.	Survived.
	cc.			
140 + 1,400	0.0001	5	0	5
140 + 2,800	0.001	5	0	5

In the following experiment three or four doses of antigen were given 3 to 4 days apart and a still higher grade of immunity was produced in the mice.

Experiment 10.—

Dose of antigen in millions of pneumococci.	Culture.	No. of mice.	Died.	Survived.
	cc.			
175 + 350 + 700	0.001	5	0	5
175 + 350 + 700 + 1,400	0.01	5	2	3
175 + 350 + 700 + 1,400	0.02	4	1	3

A single dose of antigen, even when very large, was not so effective as several smaller doses.

Experiment 11.—

Dose of antigen in millions of pneumococci.	Culture.	No. of mice.	Died.	Survived.
	cc.			
5,600	0.01	3	2	1
5,600	0.05	3	3	0
8,500	0.001	2	2	0
17,000	0.001	2	2	0
25,500	0.001	2	2	0

These experiments show that with several smaller injections of this antigen immunity against as much as 0.02 cc. of culture of virulent Pneumococcus Type I could be obtained. The degree of the immunity can be appreciated by the realization that 0.02 cc. of the culture was equivalent to several hundred thousand lethal doses. The single administration of one comparatively large amount induces practically no protection, as shown in Experiment 11.

In order to "deproteinize" the acid filtrate of the tryptic digest further, a portion of the latter was added to ten volumes of absolute alcohol. The resulting precipitate was collected by centrifugalization, washed in alcohol, and redissolved in physiological salt solution. This solution gave typical proteose reactions but contained no coagulable protein. When administered to mice subcutaneously in two doses it failed to protect them even against small amounts of pneumococcus culture.

Experiment 12.—

Dose of antigen in millions of pneumococci.	Culture.	No. of mice.	Died.	Survived.
	cc.			
100 + 5,000	0.00001	3	3	0
100 + 5,000	0.0001	3	3	0

On the other hand, the alcoholic filtrate, when evaporated in vacuum at 35–40°C. to dryness and redissolved in saline afforded considerable protection to mice, as great as the ordinary vaccine of heat-killed pneumococci.

Experiment 13.—

Dose of antigen in millions of pneumococci.	Culture.	No. of mice.	Died.	Survived.
	cc.			
100 + 5,000	0.001	3	0	3
100 + 2,000 + 10,000	0.01	3	1	2
100 + 2,000 + 10,000	0.1	3	1	2

This antigen gave no cross-immunity against a culture of virulent Pneumococcus Type II.

Experiment 14.—

Dose of antigen in millions of pneumococci.	Culture of Pneumococcus Type II.	No. of mice.	Died.	Survived.
	cc.			
100 + 2,000 + 10,000	0.00001	3	3	0

The alcohol-soluble fraction of the trypsin digest of which only a very small amount was available for chemical study contained but little nitrogen (5 mg. per 100 cc. of solution, 1 cc. of which was equivalent to 80 billion pneumococci), gave a negative biuret test, and positive Millon, xanthoproteic, and ninhydrin tests, showing the presence of aromatic (cyclic) amino-acids, and the absence of higher polypeptides, peptone, and proteoses. It is generally conceded that the amino-acids and lower polypeptides do not act as antigens in any sense. One is forced then to assume that the antigenic factor in the alcohol-water-soluble fraction is a non-protein, non-lipin substance.

That the antigen is not of lipoidal or fatty nature was demonstrated by thoroughly extracting the acid filtrate of the trypsin digest with ether for several days. The alcohol-soluble fraction of the trypsin digest was treated in the same manner. These lipin-free antigen solutions conferred a degree of immunity equal to that of the unextracted antigen solutions.

Experiment 15.—

Dose of antigen in millions of pneumococci.	Culture.	No. of mice.	Died.	Survived.
	cc.			
100 + 5,000	0.001	3	0	3
100 + 5,000	0.01	3	0	3
100 + 5,000	0.1	3	1	2

In this connection it may be mentioned that alcohol, ether, chloroform, and acetone extracts were made from intact dried pneumococci, and sodium salts were prepared from the fatty acids of pneumococci in accordance with Warden's method (11). Repeated attempts to immunize mice with these fatty and lipoidal fractions failed. These sodium salts of fatty acids obtained from Pneumococcus Type I were dissolved in Ringer's solution and cholesterol was added. Warden found in his experiments that cholesterol reinforces the antigenic value of the bacterial fats.

Experiment 16.—Six mice were each injected subcutaneously with three doses of a solution of a sodium salt of fatty acids from Pneumococcus Type I reinforced with cholesterol. The amount given in each injection was 0.5 cc., containing 0.25 mg. of soap and 1 mg. of cholesterol. At the same time three control mice were injected with three doses of a solution of cholesterol, each dose containing 1 mg. of cholesterol. 9 days after the last injection the vaccinated mice and the control mice were each inoculated intraperitoneally with 0.0000001 cc. of a broth culture of virulent Pneumococcus Type I. All the mice died in 48 hours.

Experiment 17.—Antigen prepared as in the preceding experiment. Feb. 7, 1921. Five mice received each 0.5 cc. of the antigen subcutaneously, containing 1 mg. of sodium soaps from Pneumococcus Type I and 1.25 mg. of cholesterol. The second, third, and fourth doses given subcutaneously on Feb. 10, 12, and 15 respectively contained the same amount of antigen and cholesterol as the first dose. Feb. 23 (8 days after the last injection). The five vaccinated mice and a control mouse were each inoculated intraperitoneally with 0.0000001 cc. of a broth culture of virulent Pneumococcus Type I. All the mice died.

These experiments show that immunity could not be obtained in white mice by subcutaneous injections of salts of fatty acids derived from Pneumococcus Type I even against a very small infecting dose of the homologous organism.

IV. Behavior of Pneumococcus Antigen towards Heat, and Its Keeping Qualities.

Since it was not deemed desirable to introduce preservatives and the uncertainty attendant upon their use with antigen solutions, also in order to test the thermostability of the antigen, the following experiments on the effect of sterilization by heat were performed. Two portions of the acid filtrate of tryptic digests of pneumococci, which contained antigen as shown in Experiment 9, were heated in test-tubes of $\frac{1}{2}$ inch diameter for 5 minutes by immersion in boiling water. The reaction in one tube was kept at its original acidity of approximately pH 6.0, while the contents of the second tube were made alkaline to pH 9.0 with dilute sodium hydroxide. In the acid solution a slight precipitate was produced on heating, whereas the alkaline solution remained clear. After cooling, both solutions were filtered through Mandler filters and clear filtrates were obtained. The acid filtrate gave a negative biuret reaction and contained 21 mg. of nitrogen per 100 cc., while the alkaline filtrate gave a positive biuret reaction and contained 38.5 mg. of nitrogen per 100 cc. Four mice were used for testing each of these two solutions by the method given in preceding experiments.

Experiment 18.—Antigen injections 4 days apart. Inoculation with pneumococcus culture 10 days after last dose of antigen.

Antigen in millions of pneumococci.	Culture.	No. of mice.	Died.	Survived.
	cc.			
Boiled acid antigen.				
100 + 2,000 + 10,000	0.001	4	0	4
Boiled alkaline antigen.				
100 + 2,000 + 10,000	0.001	4	4	0

This experiment shows that boiling the antigen solution for 5 minutes in a slightly acid medium does not destroy its immunizing property, and that boiling the same solution for the same length of time but in alkaline solution appears to destroy the antigen. This single experiment also seems to indicate that the coagulable protein occasionally remaining in the tryptic digest is not the antigenic factor.

In another series of experiments (Experiments 19 and 20) an acid filtrate of a tryptic digest of Pneumococcus Type I and a solution of the alcohol-soluble fraction were used separately for immunization

experiments on mice 1 month after preparation. A portion of each solution with a pH of 6.0 was heated in a water bath at 56°C. for 1 hour. The heated and the unheated solutions were then left in a refrigerator at 4°C. without any preservative for 2½ months and at the end of that period their antigenic power was again tested on mice. In each step of these two experiments the mice received the same quantity of antigen in the same number of doses. Three doses were given 4 days apart. The amounts given were the equivalents of 50, 2,000, and 10,000 million respectively of killed Pneumococcus Type I. 10 days after the last injection of antigen the mice were inoculated intraperitoneally with a culture of virulent Pneumococcus Type I.

Experiment 19.—

Antigen.	Culture.	No. of mice.	Died.	Survived.
	cc.			
Acid filtrate of tryptic digest, kept 1 mo.	0.000001	6	0	6
	0.00001	3	2	1
	0.0001	3	1	2
" " " " " heated at 56°C. for 1 hr. and kept for 2½ mos. after heating.	0.000001	6	2	4
	0.00001	3	0	3
	0.0001	3	1	2
Acid filtrate of tryptic digest, unheated, kept for 3½ mos.	0.000001	5	0	5
	0.00001	3	2	1
	0.0001	3	1	2

Experiment 20.—

Antigen.	Culture.	No. of mice.	Died.	Survived.
	cc.			
Alcohol-soluble fraction of tryptic digest, kept 1 mo.	0.000001	5	0	5
	0.00001	3	2	1
	0.0001	2	2	0
Alcohol-soluble fraction of tryptic digest, heated at 56°C. for 1 hr. and kept for 2½ mos. after heating.	0.000001	5	3	2
	0.00001	3	1	2
	0.0001	3	3	0
Alcohol-soluble fraction of tryptic digest, unheated, kept for 3½ mos.	0.000001	6	3	3
	0.00001	3	2	1
	0.0001	3	1	2

These experiments demonstrate that the slightly acid antigenic solutions may be kept in the refrigerator for at least 3 months and that they may be heated for fully 1 hour at 56°C. without deterioration or decrease in their immunizing property. The question of the duration of the immunity produced in mice by the various antigens is being investigated.

After the procedure for extracting the antigen had been worked out it was observed that it resembled in many respects that of Wildiers' (12) for the preparation of his now classic bios, or growth-promoting factor in yeast, especially as regards resistance to autolytic digestion and solubility in 80 per cent alcohol. This analogy suggested experiments which were carried out with some of the antigenic solutions.

Experiment 21.—0.00000001 cc. (in 1 cc.) of an 18 hour plain broth culture of Pneumococcus Type I was added to each of two tubes containing 10 cc. of plain broth and 0.2 cc. of pneumococcus antigen. Two tubes, each containing 10 cc. of plain broth and 0.2 cc. of 0.9 per cent NaCl were inoculated with 0.00000001 cc. of the culture at the same time and acted as controls. All the tubes were incubated for 6 hours at 37.5°C. At the end of this period the growth was determined by pouring blood agar plates with 1 cc. of the culture and colony count after 18 hours incubation.

Plate No.	Culture and antigen.	Colonies.
1	0.00000001 cc. of culture with 0.2 cc. of antigen.	415
2	0.00000001 " " " " 0.2 " " "	620
3	0.00000001 " " " " 0.2 " " saline.	4
4	0.00000001 " " " " 0.2 " " "	15

The experiment was repeated with the same Pneumococcus Type I antigen with 0.0000001 cc. of a broth culture of Pneumococcus Type II, also with 0.000001 cc. of a broth culture of *Streptococcus hæmolyticus*. In both instances the same remarkable growth-promoting effect was observed. While the number of observations on this phase of our work is still insufficient to warrant definite conclusions, the few experiments described indicate that a non-specific growth-promoting agent for bacteria is extracted along with the antigen. This interesting similarity of two apparently different biological agents may lead to a clearer understanding of the ultimate chemical nature of bacterial antigen. Further research on this aspect of the problem is in progress.

V. Experiments with Antigens Prepared from Pneumococcus Types II and III.

From the experiments which have been presented above it will be seen that water-soluble extracts of pneumococcus antigen can readily be prepared from Pneumococcus Type I by several different methods. It seemed desirable to determine whether similar antigens could be prepared from the other two fixed types of pneumococcus. In the following experiment the antigen was prepared from a virulent Pneumococcus Type II.

Experiment 22.—White mice were injected subcutaneously with a saline suspension of Pneumococcus Type II killed by heating at 60°C. for 1 hour. Six mice were injected subcutaneously each with 100 million (0.5 cc.) Pneumococcus Type II saline vaccine. 6 days later the mice received each 1,000 million (0.5 cc.) Pneumococcus Type II saline vaccine. 11 days after the second injection the six vaccinated mice and two control mice were each inoculated intraperitoneally with a broth culture of virulent Pneumococcus Type II.

No. of mice.	Culture.	Died.	Survived.
	cc.		
3	0.00001	1	2
3	0.001	1	2
1 (control).	0.00000001	1	0
1 (").	0.0000001	1	0

The results of this experiment show that immunity against Pneumococcus Type II can be obtained by two subcutaneous injections of a saline suspension of killed Pneumococcus Type II. In the following experiment an antigen consisting of an acid filtrate of tryptic digest of Pneumococcus Type II was used. The antigen was prepared from a portion of the same suspension of Pneumococcus Type II that was used in Experiment 22.

Experiment 23.—Six mice were each injected subcutaneously with three doses of antigen at 4 day intervals. The amounts given were the equivalents respectively of 100, 2,000, and 10,000 million killed pneumococci. 2 days after the last injection of antigen three of the vaccinated mice and control mice were inoculated intraperitoneally with a broth culture of virulent Pneumococcus Type II. The remaining three vaccinated mice and control mice received the virulent culture on the 6th day after the last dose of antigen.

Period after last dose of antigen.	Culture.	No. of mice.	Died.	Survived.
days	*cc.*			
2	0.00001	3	2	1
6	0.0001	3	2	1
Control.	0.00000001	1	1	0
"	0.0000001	1	1	0
"	0.000001	1	1	0

Experiment 24.—In this experiment the alcohol-soluble fraction of a tryptic digest of Pneumococcus Type II was used as an antigen. Six mice received each three doses of this antigen subcutaneously. The injections were given 3 to 4 days apart and the amounts were the equivalents of 100, 2,000, and 10,000 million respectively of killed pneumococci. 6 days after the last injection of the antigen three of the vaccinated mice with controls were inoculated intraperitoneally with a culture of virulent Pneumococcus Type II. The remaining three vaccinated mice were inoculated intraperitoneally with Pneumococcus Type II 11 days after the last dose of antigen.

Period after last dose of antigen.	Culture.	No. of mice.	Died.	Survived.
days	*cc.*			
6	0.00001	3	1	2
11	0.0001	3	2	1
Control.	0.00000001	1	1	0
"	0.0000001	1	1	0

Experiments 23 and 24 show that the filtrates of trypsin digests of Pneumococcus Type II and the alcohol-soluble fraction of these digests confer immunity in mice against Pneumococcus Type II.

Finally, the antigenic power of Pneumococcus Type III was studied. The first experiments with antigens from Pneumococcus Type III were carried out with saline suspensions of killed pneumococci.

Experiment 25.—Twelve mice were each injected subcutaneously with three doses of a saline suspension if Pneumococcus Type III, killed by heating at 60°C. for 1 hour. The injections were given 4 days apart, the doses being 100, 1,000, and 2,000 million respectively. 10 days after the last injection of vaccine the mice and controls were inoculated intraperitoneally with a broth culture of virulent Pneumococcus Type III.

No. of mice.	Culture.	Died.	Survived.
	cc.		
3	0.000001	1	2
3	0.00001	1	2
3	0.0001	1	2
2	0.001	2	0
2 (control).	0.00000001	2	0
2 (").	0.0000001	2	0

This experiment shows that a definite immunity can be obtained against Pneumococcus Type III by three subcutaneous injections of the saline suspension. In the following experiment the antigen used was an alcoholic extract of a trypsin digest of Pneumococcus Type III prepared according to the technique described previously.

Experiment 26.—Six mice were each injected subcutaneously with three doses of an alcoholic extract of a trypsin digest of Pneumococcus Type III antigen. The injections were given at intervals of 4 days and the quantities injected were equivalent to 50, 500, and 5,000 million respectively of killed pneumococci. 7 days after the last injection of antigen the treated mice and control mice were inoculated intraperitoneally with a broth culture of virulent Pneumococcus Type III.

No. of mice.	Culture.	Died.	Survived.
	cc.		
3	0.000001	1	2
3	0.0001	2	1
1 (control).	0.00000001	1	0
1 (").	0.0000001	1	0

As in the case of Pneumococcus Types I and II, so with Pneumococcus Type III, an alcoholic extract of a trypsin digest of the bacteria conferred an immunity against the homologous type, approximately as high as that obtained by the original saline vaccine.

DISCUSSION.

From the experiments which have been reported in this paper it is clear that the immunizing antigen can be isolated from the three fixed types of pneumococcus by comparatively simple chemical methods.

This antigen in its purified form contains only a trace of nitrogen. In the alcoholic extract of a tryptic digest the nitrogen determination showed only 5 mg. of nitrogen per 100 cc. of antigen.

While on the basis of the reported experiments it cannot be definitely stated that the immunizing antigen is non-protein in nature, the evidence presented points strongly in that direction. The solubility in 90 per cent alcohol taken in conjunction with the remarkable heat stability and the resistance to the prolonged action of proteolytic enzymes does establish beyond reasonable doubt that the substance is much simpler in nature than the large and complex molecule of native bacterial protein.

It will be recalled in this connection that the purified pneumococcus antibody obtained recently by Huntoon (13) possessed similar chemical properties. The only notable bacterial antigen possessing properties almost identical with those of our antigen is the "Coagulin B," obtained by Pick (14), which was a saline extract of young cultures of typhoid bacilli. It gave a specific precipitate with homologous immune serum, was protein-free, soluble in alcohol, and resistant to heat and to proteolytic enzymes. This striking parallel came to the attention of the authors after the completion of the experimental work herein described and it served to strengthen their belief in the non-protein nature of the immunizing pneumococcus antigen.

The chemical data pertaining to the constitution of the antigen are mainly negative, resembling in this respect the findings concerning the ultimate composition of two other large classes of biological agents, the ferments and the vitamines. But unlike these last two the chemistry of the bacterial antigens has not been subjected to intensive study.

The relation which the immunizing antigen described in these experiments may bear to the other antigens contained in the pneumococcus, *e.g.* precipitinogen, agglutinogen, and complement fixation antigen, is of obvious interest, and the authors hope to report some interesting data on this subject in the near future. The recent study by Zinsser and Parker (15) renders the further investigation of the relation existing between the various antigens all the more interesting and urgent. It is quite significant that the antigen extracted by Zinsser and Parker from pneumococci by weak alkaline solutions and

deproteinized by acidification and heat yielded a non-protein substance, similar to Pick's "coagulin" (14), which produced specific precipitates with homologous serum, but on the other hand produced no antibodies when injected into animals. It is to be noted that while this substance resembled the antigen described in the present paper in its resistance to heat, it differed from it in being precipitable by alcohol. The immunizing antigen herein described was freely soluble in 90 per cent alcohol and the alcohol-insoluble fraction after careful washing with 90 per cent alcohol contained no immunizing antigen (Experiment 12). These results strongly suggest that the precipitinogen and the immunizing antigen are distinct entities. It may be stated that among the various antigenic solutions tested with immune serum the writers found a number which produced specific precipitates yet failed to immunize mice; on the other hand, some of the potent alcohol-soluble immunizing antigens failed to produce precipitates with homologous serums.

These purified pneumococcus antigens are non-toxic for mice and could probably be used in comparatively large doses in man without exciting severe local or general reactions.

CONCLUSIONS.

1. Active immunity against many lethal doses of Pneumococcus Types I, II, and III may be produced in mice by two or three subcutaneous injections of the homologous type of pneumococcus saline vaccine.

2. Mice may be actively immunized with the protein fraction obtained by treating pneumococci with anhydrous sodium sulfate or by solution of pneumococci in bile salts and precipitation with alcohol. Pneumococcus antigen is therefore carried within or adheres to the protein fraction of the organism.

3. Pneumococcus antigen is resistant to prolonged autolysis and to tryptic digestion, and can be recovered from the soluble portions of digests of either the intact bacteria or the bacterial protein.

4. The antigen may be isolated from each of the three fixed types of pneumococcus by tryptic digestion of the pneumococci and extraction of the digest with 70 to 90 per cent alcohol. The antigen is not soluble in absolute alcohol, nor is it soluble in ether or in the other lipoidal solvents.

5. The immunizing property of slightly acid solutions of the antigen is not impaired by boiling for 5 minutes, nor by heating at 56°C. for 1 hour. Sterile unpreserved solutions of the antigen did not deteriorate by standing in the refrigerator for $3\frac{1}{2}$ months.

6. The exact chemical nature of pneumococcus antigen still remains to be determined. From the experiments reported it appears that the antigen is non-lipoidal, that it probably adheres to the protein fraction in a loose chemical or physical union rather than representing a protein complex of a large molecular size, as shown by its solubility in alcohol, its thermostability, and its resistance to proteolytic digestion.

7. Some of the antigens studied have been shown to contain a non-specific factor promoting the growth of bacteria.

8. The purified pneumococcus antigen solutions are non-toxic for mice.

BIBLIOGRAPHY.

1. Cecil, R. L., and Steffen, G. I., *J. Exp. Med.*, 1921, xxxiv, 245; 1923, xxxviii, 149.
2. Wadsworth, A. B., *J. Immunol.*, 1920, v, 429.
3. Much, H., *Münch. med. Woch.*, 1920, lxvii, 1005.
4. Rowland, S., *J. Hyg.*, 1912, xi, suppl. 1, pp. 11, 20; xii, suppl. 2, pp. 340, 344, 350, 358; 1914, xiii, suppl. 3, pp. 403, 418, 440.
5. Rosenow, E. C., *J. Infect. Dis.*, 1911, ix, 190; 1912, x, 113; 1912, xi, 94, 235, 286; *J. Am. Med. Assn.*, 1918, lxx, 759.
6. Jobling, J. W., and Strouse, S., *J. Exp. Med.*, 1912, xvi, 860.
7. Hirschfelder, J. O., *J. Am. Med. Assn.*, 1912, lix, 1373.
8. Osborne, T. B., Mendel, L. B., and Harris, I. F., *Am. J. Physiol.*, 1905, xiv, 259.
9. Greenwald, I., *J. Biol. Chem.*, 1915, xxi, 61.
10. Folin, O., and Farmer, C. J., *J. Biol. Chem.*, 1912, xi, 493.
11. Warden, C. C., *J. Infect. Dis.*, 1918, xxii, 133; xxiii, 504; 1919, xxiv, 285.
12. Wildiers, *La Cellule*, 1900, xvii, 385.
13. Huntoon, F. M., *J. Immunol.*, 1921, vi, 117. Huntoon, F. M., and Etris, S., *J. Immunol.*, 1921, vi, 123. Huntoon, F. M., Masucci, P., and Hannum, E., *J. Immunol.*, 1921, vi, 185.
14. Pick, E. P., *Beitr. chem. Physiol. u. Path.*, 1902, i, 397; also discussed in detail by Paltauf (Paltauf, R., in Kolle, W., and von Wassermann, A., Handbuch der pathogenen Mikroorganismen, Jena, 2nd edition, 1913, ii, 567).
15. Zinsser, H., and Parker, J. T., *J. Exp. Med.*, 1923, xxxvii, 275.

CONDITIONS DETERMINING THE TRANSPLANTABILITY OF TISSUES IN THE BRAIN.

By JAMES B. MURPHY, M.D., and ERNEST STURM.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

Plates 13 to 16.

(Received for publication, March 14, 1923.)

The study of the transplantability of animal tumors is largely responsible for the view that it is impossible among warm blooded animals to graft tissues from one species to another, and that even grafting within a species has definite limitations. Leo Loeb,[1] among others, has brought forward evidence indicating that the conditions governing the transplantability of tumors are applicable essentially to normal tissues, and Rous[2] has shown that the methods of inducing resistance to tumor transplantation apply also to normal tissue grafts. In his well known zig-zag experiments, Ehrlich[3] showed that the limit of growth of a tumor graft in a foreign species (rat tumors in mice, or *vice versa*) is from 7 to 9 days, but in no instance has such a graft been successfully implanted into a second individual of the foreign species without being first returned to the original or homologous species. One of us demonstrated that this restriction of growth does not apply to the embryo, since rat tissues are capable of growing over long periods and through successive transplantation in chicken embryos.[4]

The explanation of the strict specificity of tissue transplantability has been sought in various ways and led to not a few hypothetical views. A certain number of indubitable facts have been discovered, but whether or not they are to be regarded as the essential or primary

[1] Loeb, Leo, *Proc. Am. Phil. Soc.*, 1908, xlvii, 3; *J. Cancer Research*, 1917, ii, 135; *J. Med. Research*, 1920–21, xlii, 137.

[2] Rous, P., *J. Exp. Med.*, 1910, xii, 344.

[3] Ehrlich, P., *Arb. k. Inst. exp. Therap. Frankf.*, 1906, No. 1, 77.

[4] Murphy, Jas. B., *J. Exp. Med.*, 1913, xvii, 482.

cause of the phenomenon or merely the secondary or concomitant and attendant results has not yet been determined.

Thus, it has been noted that a short time after the implantation of a heteroplastic graft, the latter becomes surrounded by a zone of cells in which the lymphoid variety of cell is dominant; the intensity of the cellular reaction is roughly proportionate to the degree of relationship existing between the two species concerned in the experiment.

On the other hand, it has been found that with the rat tumor graft in chicken embryos, referred to above, the cellular reaction is much delayed and does not become evident until the 19th day of incubation and coincides with the appearance of degenerative changes in the graft, which now suffers rapid and ultimately complete absorption.[5] It is significant that the cellular reaction about, and subsequent disintegration of the graft are hastened by the implantation into the embryo of a fragment of spleen from an adult chicken.[6]

These findings indicate unmistakably that the lymphoid cell reactions are associated with the resistance mechanism, and the importance of this association is emphasized by the fact that adult animals deprived of their lymphoid tissue by repeated exposures to suitable small doses of x-rays, like the embryo, fail to destroy foreign tissue grafts, which not only continue to grow actively, but may be transferred repeatedly to other individuals which have been prepared for their reception by the x-ray treatment.[7] One would expect that an animal with its resistance so reduced that even inoculated foreign tissue survives and grows actively would prove to be non-resistant to transplants from homologous spontaneous tumors, but this is not the case. Grafts from a spontaneous tumor undoubtedly survived for a longer period in x-rayed than in untreated animals, but we did not succeed in transplanting a tumor, under these conditions, which failed to grow in untreated or normal animals. One instance has been reported of transplantation of a rat tumor in x-rayed animals which failed in untreated rats,[8] but this method falls far short of what might be expected.

[5] Murphy, Jas. B., *J. Exp. Med.*, 1914, xix, 181.
[6] Murphy, Jas. B., *J. Exp. Med.*, 1914, xix, 513.
[7] Murphy, Jas. B., *J. Am. Med. Assn.*, 1914, lxii, 1459.
[8] Chambers, H., Scott, G., and Russ, S., *J. Path. and Bact.*, 1920, xxiii, 384.

The site of tissue transplanation has received but little attention, and as a rule the experimental studies have been made on grafts in the subcutaneous tissue or muscle. New points for consideration have been introduced by recent experiments reported by Shirai,[9] who has employed the brain as the locus of heteroplastic tumor transplants, in which situation, according to him, grafted tissue grows as readily in an alien as in an homologous host. The important bearing of this observation on the question of the resistance mechanism has led us to undertake an investigation of the subject.

Heterologous Tumor Grafts in the Brain.

Since Shirai has not described adequately the procedure he employed, we had first to work out a practical method.

Method.—After etherization of the animal, the head is shaved and a midline incision made, enabling the skin and fascia to be retracted. A small hole of sufficient size to admit a No. 18 gauge trocar is made in the skull. The trocar is provided with a shoulder of metal about 3 mm. from the point, in order to limit the depth of penetration. The point is beveled slightly so as not to cause unnecessary damage to the brain tissue. The material for inoculation is loaded into the trocar and pushed into the brain by means of a plunger, after which the trocar is withdrawn slowly so as not to remove the graft. Bleeding may be stopped by pulling the fascia over the opening or by applying a small piece of muscle.

Mouse Tumors in the Brains of Rats.—The cerebellum was chosen as the location for the first inoculations, but for reasons not altogether clear, this proved to be unsatisfactory. Occasionally excellent growths resulted, but in many animals the graft remnant was found imbedded in a mass of reaction tissue similar to that which appears about an heteroplastic graft in the subcutaneous tissue. Implantations into the posterior lateral part of the cerebrum also failed to give uniform results, because as was found, a graft lying in the ventricle or even coming into contact with the ventricle leads to a similar reaction. On the other hand, when the graft lay entirely in the brain substance, it grew almost invariably with great rapidity.

This observation led us to select the anterior part of the frontal lobe on account of the thickness of the cortex and the shallowness of the ventricle at this point. Grafting in this region gave 80 to 90 per cent

[9] Shirai, Y., *Japan Med. World*, 1921, i, 14, 15.

of tumors in the animals inoculated. In practically every instance in which the implant failed to grow, examination showed that it had come into contact with the ventricle.

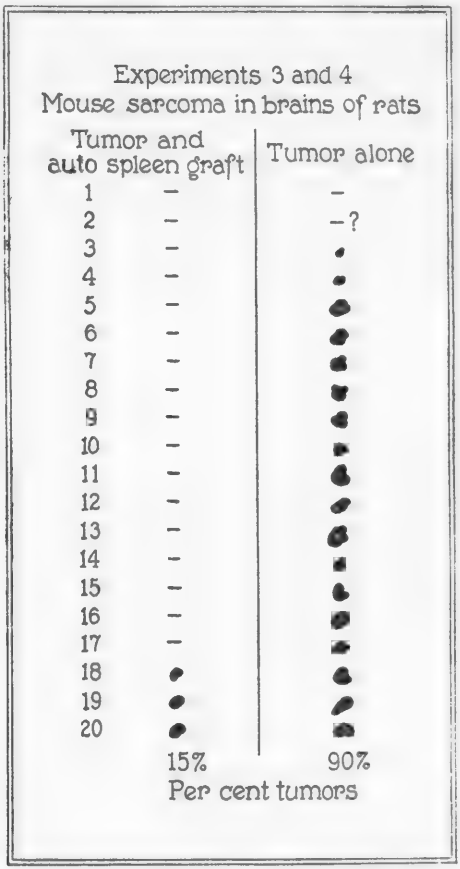
The growth resulting from inoculation of the rat brain with mouse sarcoma is generally a discrete, rounded nodule, pushing the brain substance away, rather than invading it directly. The tumors are copiously supplied with blood vessels and rarely show considerable areas of necrosis, which is in contrast with nodules developing in the subcutaneous tissue of the native host, in which the entire center of the growth is often necrotic. The number of mitotic figures is remarkable (Fig. 1). No cellular reaction is present about the edges of the typical growth lying entirely in the brain substance, but nearby vessels often show a pronounced collar of round cells (Fig. 2), and the lumina of small vessels are often blocked with lymphocytes. Other than exceptions to be mentioned, the cellular accumulations remain confined, and do not invade the brain tissue. If, in the course of growth, the tumor reaches the ventricle, the choroid plexus becomes enormously swollen and engorged with lymphocytes (Figs. 3 and 4). Under these circumstances the cells frequently invade the tumor, and necrosis of the portion of the tumor lying near the ventricle takes place (Figs. 5 and 6).

The mouse sarcoma used in these experiments grows with great rapidity in the brains of rats,—pin-head sized grafts producing tumors in 7 to 9 days which replaced the entire frontal lobe. This mouse tumor has also been grown in the brains of guinea pigs and pigeons. Moreover, a mouse carcinoma has been successfully implanted in the brains of rats, but the kind of growth obtained is somewhat different in that the tumors show a tendency to flatten on the surface of the brain and to extend apparently by invasion, sending finger-like processes into the brain (Fig. 7). In addition, necrosis is observed more frequently in the carcinoma than in the sarcoma, although less in extent than occurs in the subcutaneus tumor of the native host.

Effect of Autografts of the Spleen on the Growth of Foreign Tissue in the Brain.

As previously noted, an organism which is non-resistant to heteroplastic tissue, like the chick embryo, may be rendered resistant by a

graft of adult spleen. This, with the other evidence associating the lymphoid cell with the resistance mechanism, leads one to consider the possible importance of the observation that no cellular reaction



TEXT-FIG. 1.

occurs about a foreign tissue in the brain, and the following experiments were therefore undertaken to throw some light on this question.

Method.—The tip of the spleen of a rat was removed under ether anesthesia. and a small bit of the tissue, together with a graft of a mouse sarcoma, was im-

planted into the brain of the same rat. As a control, a graft of the tumor alone was introduced into the brain of another rat.

Of 50 rats receiving a graft of mouse sarcoma and an autograft of spleen, only eight developed tumors, and practically without exception these were small, often only nests of cells. On the other hand, 40 of the 48 controls developed tumors, and the growths generally replaced the entire frontal lobe of the brain (Table I and Text-fig. 1).

TABLE I.

Experiment No.	Tumor and auto spleen graft.		Tumor alone.	
	Takes.	No. of rats.	Takes.	No. of rats.
	per cent		*per cent*	
1	11.1	9	66.6	9
2	33.3	9	80.0	10
3	10.0	10	80.0	10
4	20.0	10	100.0	10
5	8.5	12	88.8	9
Average or total.....	16.0	50	83.3	48

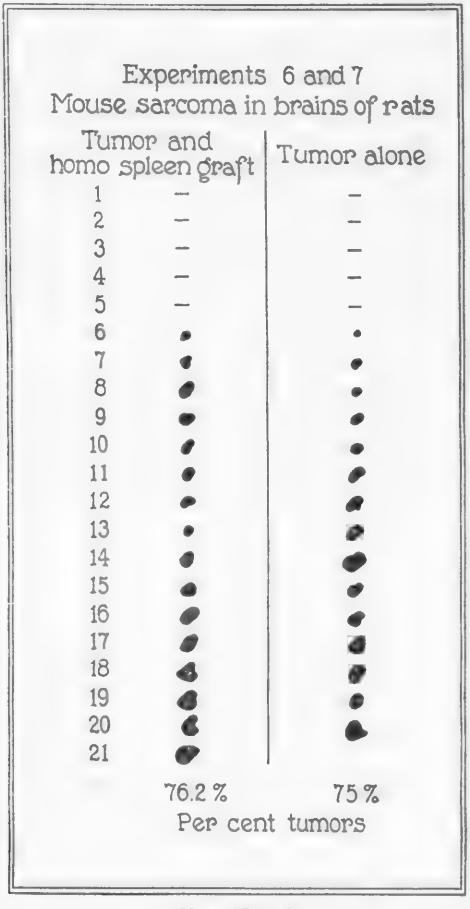
In two additional experiments, homografts of spleen instead of autografts, were introduced along with a graft of mouse sarcoma into the brains of rats, but absolutely no inhibitory action on the tumor was effected. Sixteen out of the twenty-one rats receiving tumor and homo spleen grafts developed sizable tumors, while in the controls with sarcoma alone, fifteen out of twenty had tumors (Table II and Text-fig. 2).

TABLE II.

Experiment No.	Tumor and homo spleen graft.		Tumor alone.	
	Takes.	No. of rats.	Takes.	No. of rats.
	per cent		*per cent*	
6	72.7	11	80.0	10
7	80.0	10	70.0	10
Average or total.....	76.2	21	75.0	20

This complete lack of activity on the part of the homo spleen graft is in strong contrast to the almost complete inhibitory action of the

auto spleen grafts. As a further control it was shown that autoplastic testicular grafts exert no inhibitory action, nor does autologous blood injected with the tumor affect its development.[10] In the experiment with auto spleen graft reported above, it was noted that



TEXT-FIG. 2.

the lymphoid cells disappear from the fragment very soon after the death of the tumor cells, and it has also been noted that when an auto spleen graft is inoculated alone into the brain, the lymphoid elements

[10] Maisin, J., and Sturm, E., *Compt. rend. Soc. biol.*, 1923, lxxxviii, 1216.

disappear still more rapidly. In fact it is rare to find any surviving cells of this type after 48 hours, while the cells of a similar graft in the subcutaneous tissue survive for a longer time.

Homoplastic Tumor Grafts in the Brain.

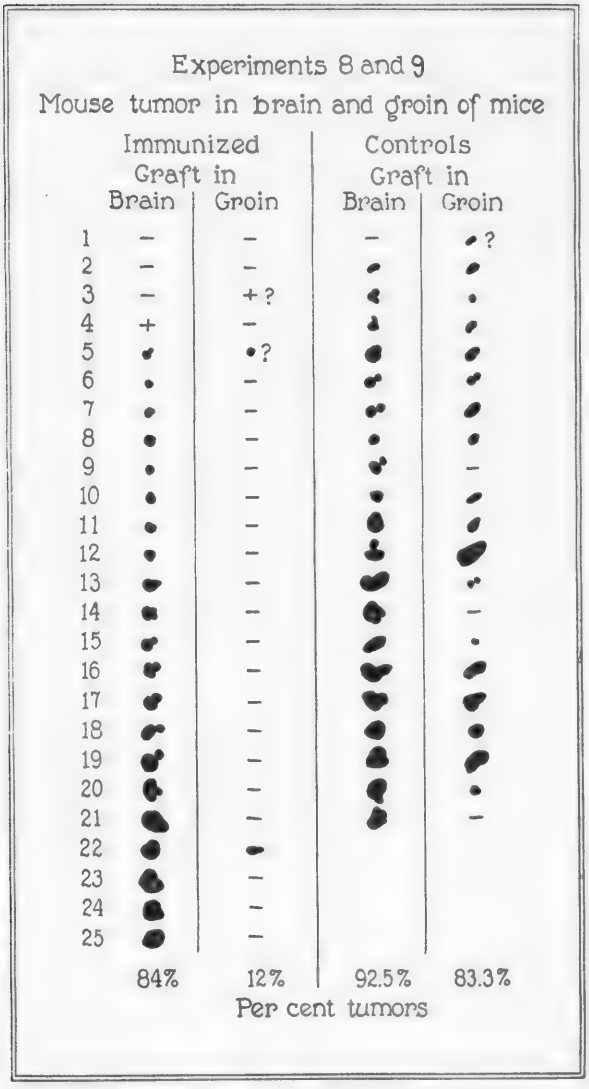
All the factors studied indicate that the mechanism involved in the destruction of the heteroplastic tissue graft is also responsible for the natural resistance possessed by a proportion of animals to homoplastic tumor grafts. Even induced resistance to tumor transplants shows itself locally by the same series of events as those which take place in natural resistance to both homo- and heteroplastic grafts. If this similarity is more than a superficial manifestation, we should expect, from the preceding observations, that animals highly resistant to homoplastic tumors, transplanted in the usual locations, would be susceptible to inoculation in the brain.

Experiment.—A number of mice were immunized against tumors by the injection of 0.2 cc. of defibrinated mouse blood, and 10 days later were inoculated with a transplantable mouse adenocarcinoma, both in the brain and in the subcutaneous tissue of the groin. As controls, normal mice were given similar inoculations with the same tumor. The results of six experiments carried out on 127 mice are given in Table III. Text-fig. 3 shows the results in two of the experiments.

TABLE III.

Experiment No.	Immunized mice.			Control mice.		
	Tumor in brain.	Tumor in groin.	No. of mice.	Tumor in brain.	Tumor in groin.	No. of mice.
	per cent	*per cent*		*per cent*	*per cent*	
8	80.0	10.0	10	100.0	77.7	9
9	86.6	13.3	15	91.6	83.3	12
10	91.6	25.0	12	72.7	81.8	11
11	88.8	12.0	9	100.0	90.0	10
12	100.0	22.0	9	100.0	80.0	10
13	90.0	30.0	10	90.0	80.0	10
Average or total.....	89.2	21.5	65	91.9	82.2	62

It may be concluded from the results of these experiments that while the immunized mice show the usual high rate of immunity against subcutaneous grafts, the same animals have almost a complete


Experiments 8 and 9
Mouse tumor in brain and groin of mice
Immunized
Graft in
Brain
Groin
Controls
Graft in
Brain
Groin
1 – – – –?
2 – – – –
3 – +? – –
4 + – – –
5 –? – –
6 – – – –
7 – – – –
8 – – – –
9 – – – –
10 – – – –
11 – – – –
12 – – – –
13 – – – –
14 – – – –
15 – – – –
16 – – – –
17 – – – –
18 – – – –
19 – – – –
20 – – – –
21 – – – –
22 – –
23 – –
24 – –
25 – –
84% 12% 92.5% 83.3%
Per cent tumors

TEXT-FIG. 3.

lack of resistance to similar grafts in the brain. Inoculations in the latter locality resulted in tumors in 89.2 per cent of the animals, which is practically the same rate as that shown in the brain of non-immunized controls and a higher rate than resulted from an inoculation in the groin of even the control mice.

The histological study of specimens from the preceding groups of animals brought out an interesting fact relating to the heteroplastic tissue experiment in that when a graft came in contact with the ventricle in a resistant mouse, a marked cellular reaction occurred, resulting in the complete or partial destruction of the tumor. On the other hand, grafts in susceptible mice frequently grew into the ventricle and invaded the choroid plexus without inducing a reaction greater than that seen about a subcutaneous graft in a susceptible animal.

In order to test the effect of auto and homo spleen grafts on the growth of tissue in the brains of immunized and non-immunized mice, a further investigation with homoplastic tissue was undertaken.

Experiment.—55 mice were immunized by the injection of 0.2 cc. of defibrinated mouse blood, and 10 days later, twenty-seven of these were inoculated in the brain with a mouse carcinoma and a bit of analogous spleen tissue. The remaining twenty-eight mice were inoculated in the brain with tumor alone. In addition, all the animals of the two groups were given a graft of tumor in the groin. As a control, normal mice were inoculated in the groin and in the brain with tumor alone.

In another experiment, normal mice were inoculated in the brain with autologous spleen grafts and tumors, and in a second series with homologous spleen and tumor. All animals received in addition a graft of the tumor alone in the groin, and control animals were also inoculated with the same tumor in the brain and in the groin. For comparison, the results of the two experiments are given in Table IV.

TABLE IV.

Experiments 14, 15, and 16.

Immunized mice.				Control mice.	
27 mice inoculated with		28 mice inoculated with		30 mice inoculated with	
Auto spleen graft and tumor in brain.	Tumor alone in groin.	Tumor alone in brain.	Tumor alone in groin.	Tumor alone in brain.	Tumor alone in groin.
per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
29.6	25.9	89.3	31.4	96.6	83.3

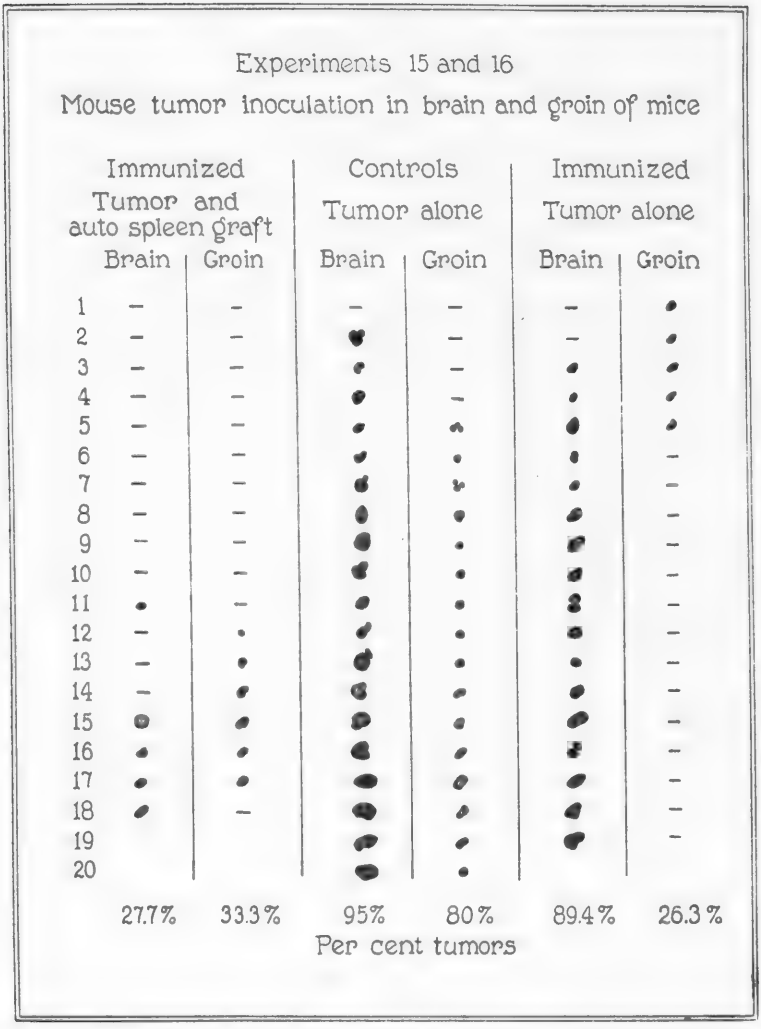
TABLE IV—*Concluded.*

Experiments 17 and 18.

Non-immunized mice.				Control mice.	
20 mice inoculated with		19 mice inoculated with		20 mice inoculated with	
Auto spleen graft and tumor in brain.	Tumor alone in groin.	Homo spleen graft and tumor in brain.	Tumor alone in groin.	Tumor alone in brain.	Tumor alone in groin.
per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
55.0	75.0	84.2	84.2	95.0	70.0

It may be deduced from the above table that immunized mice inoculated into the brain with homologous spleen and tumor grafts have considerable resistance, while immunized mice inoculated with tumor alone are almost completely lacking in a defensive reaction to tumor growth (Text-fig. 4). Even non-immunized mice, inoculated with auto spleen and tumor grafts show some resistance, but much less than that exhibited by the immunized animals. In contrast to the effectiveness of the auto spleen graft in its inhibitory action is the almost complete inactivity of homo spleen grafts. In this respect we have another agreement in the behavior of hetero- and homoplastic grafts in the brain.[11]

[11] Some time ago we carried out an experiment to test the effect of auto- and homosplenic tissue on the fate of tumor grafts inoculated in the subcutaneous tissue, the splenic tissue in this experiment being mixed with the tumor cells before inoculation. The results based on the inoculation of 114 animals were 54.6 per cent only of takes with auto- and 80 per cent of takes with homoplastic spleen, while the controls gave 94 per cent of takes. This harmonizes with the above result.


Experiments 15 and 16
Mouse tumor inoculation in brain and groin of mice
Immunized
Tumor and auto spleen graft
Brain
Groin
Controls
Tumor alone
Brain
Groin
Immunized
Tumor alone
Brain
Groin
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
27.7%
33.3%
95%
80%
89.4%
26.3%
Per cent tumors

TEXT-FIG. 4.

Failure of Grafts of Spontaneous Tumors in the Brain.

With the usual methods the spontaneous tumors of animals have proved to be rarely transplantable even to animals of the same species, and in some species no successes have been recorded. As already noted, resistance to such transplants may be somewhat mitigated by exposing the animals to x-rays, a procedure which has made it possible to transplant at least one tumor which otherwise failed, but even this method was too capricious to be of real value. The seeming complete lack of resistance of the brain naturally led to a consideration of this locality as a site for the inoculation of spontaneous tumor, with the expectation that perhaps a larger proportion might be secured for study.

Some sixty rats have been inoculated into the brain with nine different spontaneous tumors of the mouse without a single success. Over a hundred mice have been similarly inoculated with seventeen different spontaneous mouse tumors and in only ten animals could any surviving tumor cells be found at the end of 10 days or 2 weeks. In only one or two instances was there anything like a tumor formation (Fig. 8), while in the others small islands only of tumor cells imbedded in reaction tissue remained.

DISCUSSION.

It is not clear why there should be an absence of a cellular reaction about a heteroplastic graft in the brain, but two possible explanations suggest themselves; first, that for mechanical reasons the cells are unable to migrate beyond the perivascular spaces; second, that the lymphoid cells find the brain tissue an uncongenial environment. In support of the first idea are the occurrence of extensive perivascular reactions near the implant, and also the observation that the small vessels in and near the tumors are frequently crowded with, and sometimes blocked by cells of the lymphoid type. In support of the second possibility is the fact that lymphoid cells implanted alone in the brain disappear more quickly than from the subcutaneous tissue. These points are suggestive only, for we have no real knowledge of what is responsible for the absence in the brain of the usual cellular reaction about a foreign tissue graft.

There seems little doubt that the absence of a reaction of this type is the principal reason for the failure of the brain to destroy heteroplastic tissues which are so readily taken care of in other locations in the body. This notion is strengthened by the observation that a foreign tissue graft in contact with the ventricle induces the typical cellular reactions and is as promptly combated as would be a similar graft in the subcutaneous tissue. Furthermore, the necessary resistance factor may be supplied to the otherwise passive brain by the inoculation of a bit of spleen tissue along with the foreign tissue graft, this being sufficient to suppress the growth of the latter,—a condition similar to that observed in the chick embryo. On the whole, it may be said that the results described confirm and extend our earlier views on the association of cellular reaction with the resistance mechanism in respect to heteroplastic grafting.

From our previous observations it was to be expected that in the absence of cellular reactions immunity to homoplastic tumors of the transplantable type would be lacking in the brain tissues, but the explanation for the failure of spontaneous cancer grafts to grow readily even in homologous animals is not clear and must await further experimentation. It is possible, of course, that this failure may be determined as much by some inherent quality of the cancer cells as by the resistance on the part of the new host.

SUMMARY.

In confirmation of Shirai's observation, we find that transplantable mouse tumors grow actively when inoculated into the brains of rats, guinea pigs, and pigeons, whereas subcutaneous or intramuscular grafts in the same animals fail. This growth of foreign tissue in the brain, however, takes place only when the grafted material lies entirely in the brain tissue; if it comes in contact with the ventricle a cellular reaction takes place with resultant destruction of the graft.

The growth of foreign tissue in the brain may be completely inhibited by simultaneous inoculations of a small bit of autologous but not by a bit of homologous spleen tissue.

Mice highly immune to subcutaneous transplants of mouse cancer show no resistance to such tumors when the inoculation is made into the brain.

Although the brain is without obvious power of resistance to implants of transplantable heteroplastic mouse tumors, yet grafts of spontaneous tumors fail to grow there even, as a rule, when tumor implanted and animal host are of the same species.

EXPLANATION OF PLATES.

PLATE 13.

FIG. 1. A mouse sarcoma growing in the brain of a rat. *M*, mitotic figures.

FIG. 2. Perivascular infiltration in the brain of a rat, which occurs near a foreign tumor graft.

PLATE 14.

FIG. 3. Choroid plexus engorgement with round cells resulting from the encroachment of a foreign tumor.

FIG. 4. The same as Fig. 3, but showing the normal plexus at a lower part of the ventricle.

PLATE 15.

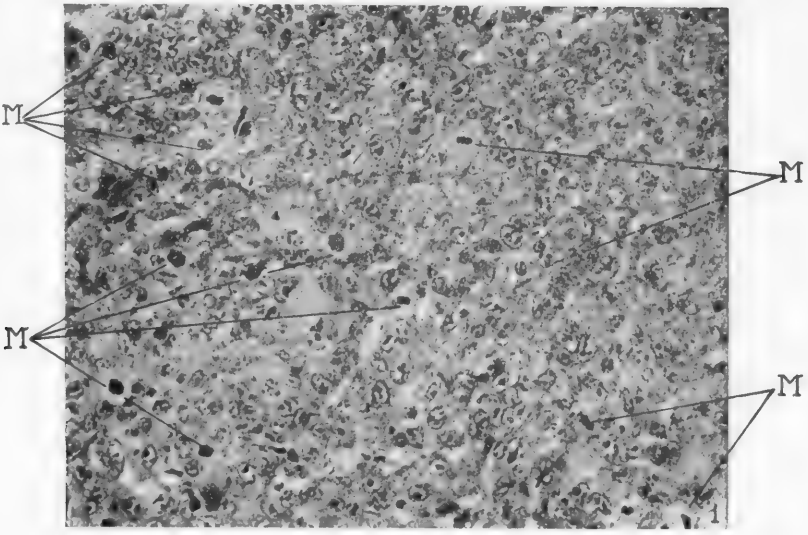
FIG. 5. Result of encroachment of a foreign tumor on the ventricle.

FIG. 6. The same as Fig. 5. The engorged choroid plexus is seen in the lower part of the section.

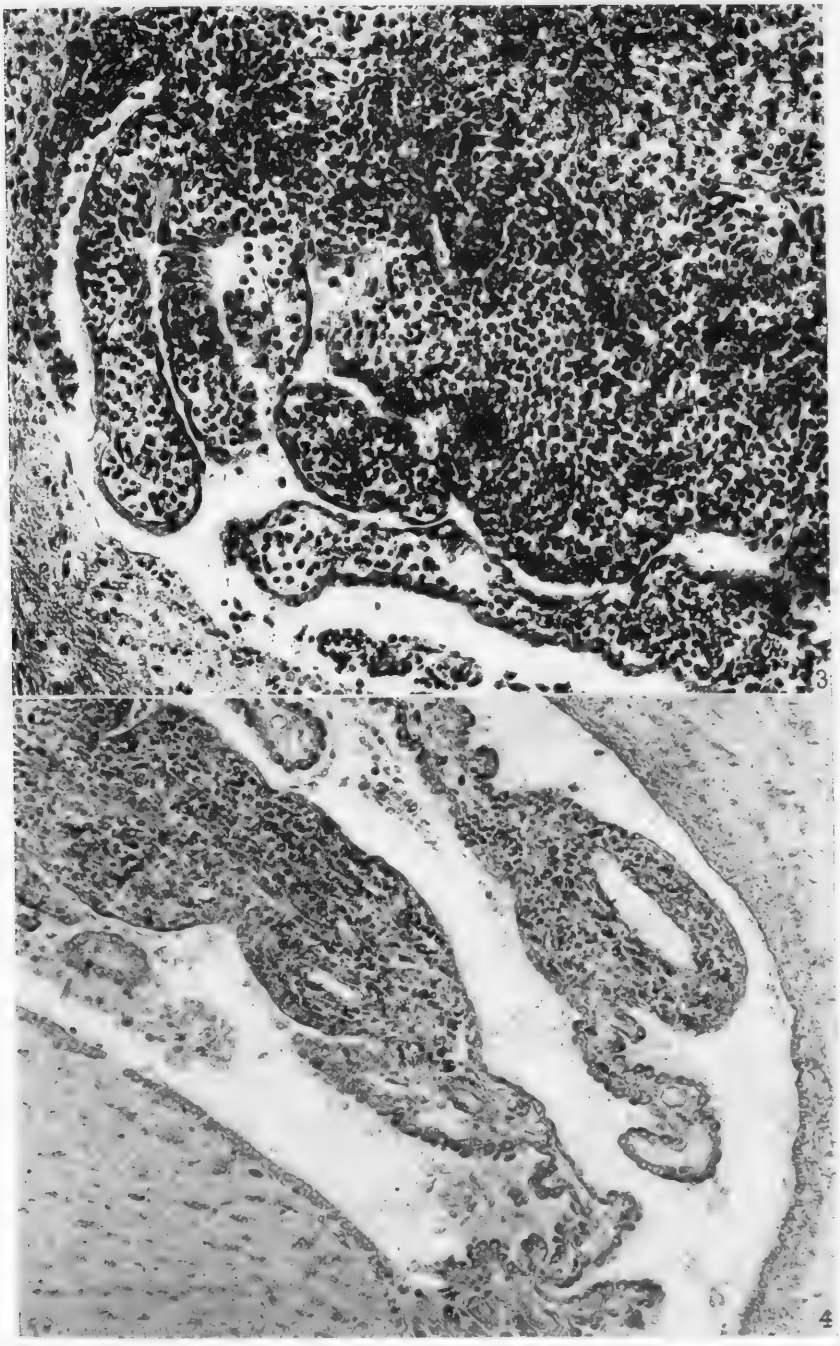
PLATE 16.

FIG. 7. The growing edge of a mouse carcinoma in the brain of a rat.

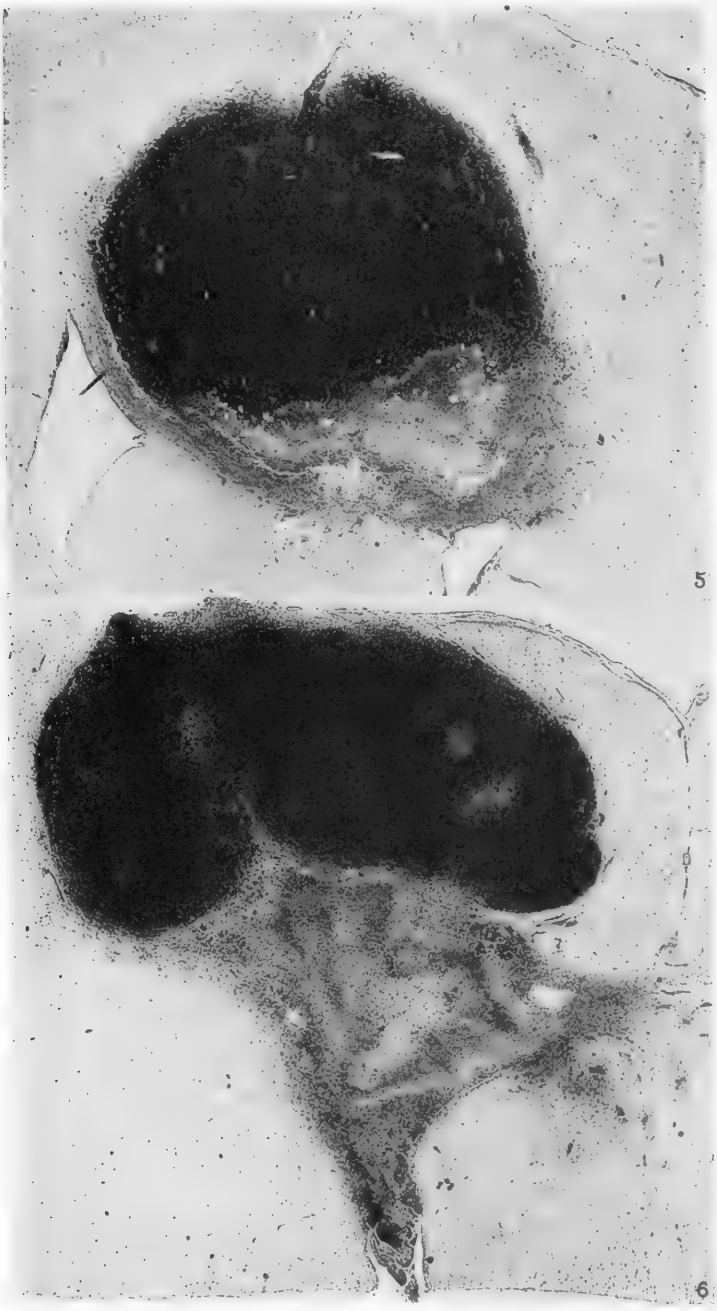
FIG. 8. Spontaneous tumor of a mouse growing in the brain of another mouse.



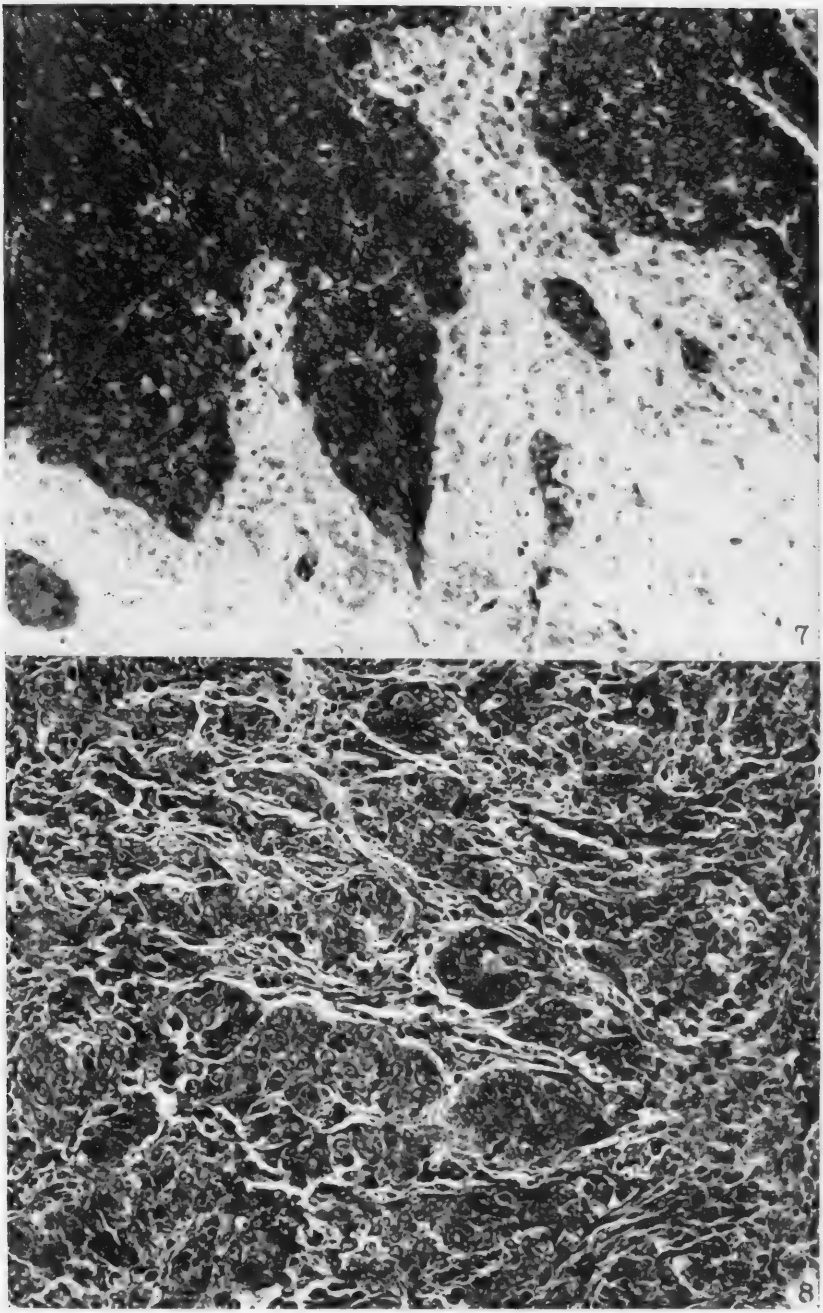
(Murphy and Sturm: Transplantability of tissues in the brain.)



(Murphy and Sturm: Transplantability of tissues in the brain.)



(Murphy and Sturm: Transplantability of tissues in the brain.)



(Murphy and Sturm: Transplantability of tissues in the brain.)

STUDIES ON THE ENZYMES OF PNEUMOCOCCUS.

IV. Bacteriolytic Enzyme.

By O. T. AVERY, M.D., and GLENN E. CULLEN, Ph.D.

(*From the Hospital of The Rockefeller Institute for Medical Research.*)

(Received for publication, April 12, 1923.)

In preceding papers (1), studies on the proteolytic, lypolytic, and carbohydrate-splitting enzymes of pneumococcus have been reported. It has been shown that these enzymes are intracellular in nature and can be extracted from the bacterial cell by various methods. They possess to a remarkable degree the power of actively hydrolyzing peptones to simpler peptides and amino-acids, of converting carbohydrates to simpler products, and of splitting esters to fatty acids.

In addition to the enzymes previously described, it has been found that solutions of the cellular substance of pneumococci also contain an enzyme which is capable of exerting a lytic action upon other dead bacterial cells.

Methods.

Preparation of Enzyme.—In previous papers, the bacteriological and chemical methods used in these studies have been described in detail (1). The same methods have been followed in the present investigation. In the preparation of active enzyme-containing solutions from pneumococci the bacterial cells have been dissolved in bile, or the intracellular substances have been extracted by autolysis. When the former method is used the presence of bile does not obscure the effects of the bacteriolytic enzyme upon heat-killed organisms inasmuch as bile itself does not cause lysis of pneumococci which have been killed by heat.

Preparation of Substrate.—The substrates have consisted of suspensions of washed bacteria in phosphate solution of known hydrogen ion concentration. The bacterial suspensions were exposed to a temperature of 60°C. for 30 minutes (water bath), or 120°C. for 20 minutes

(autoclave). Antiseptics were not used as preservatives, and the sterility of all enzyme-substrate mixtures was proved by subculture.

Action of Intracellular Enzymes of Pneumococcus on Heat-Killed Bacteria.

Experiment 1.—Enzyme: The bacterial residue from 2 liters of a 10 hour plain broth culture of Pneumoccocus Type II was washed with physiological salt solution and resuspended in 10 cc. of 0.1 M phosphate mixture of pH 6.2. The bacterial suspension was immediately frozen and thawed seven times, and kept at ice box temperature for 10 days. During this period cytolysis occurred and the bacterial detritis settled to the bottom, leaving a clear, slightly opalescent supernatant fluid which was used in this experiment. 0.1 cc. of this solution plated on blood agar showed no growth after several days incubation.

Substrate: Bacterial substrates were prepared by suspending the washed cells in 0.1 M phosphate solutions of pH 7.8. The following organisms were tested: Pneumococci Types I and II, *Streptococcus viridans*, *Streptococcus hæmolyticus*, and *Staphylococcus aureus*. The bacterial suspensions were of about equal opacity and were autoclaved 20 minutes at 15 pounds pressure before use. 0.1 cc. of the active enzyme solution was added in each instance to 0.5 cc. of the bacterial substrate, and the total volume made up to 1 cc. by the addition of 0.1 M phosphate solution of pH 7.8. The tubes were then placed in a water bath at 37°C., and the degree of bacteriolysis was noted at varying intervals by observing the relative opacity of the tubes, and by microscopic examination of stained films.

After 15 minutes incubation at 37°C., tubes containing a mixture of active enzyme and heat-killed pneumococci showed marked disintegration of the bacterial bodies. After 2 hours incubation, complete dissolution occurred and stained films showed only Gram-negative detritis, and shadow forms of the organisms. Films prepared from the tubes containing pneumococcus enzyme and the dead bodies of *Streptococcus viridans* showed at this period many Gram-negative organisms in unevenly stained chains, with disintegration of over half of the bacterial cells. On the other hand, stained films of the tubes in which the active enzyme was in contact with substrates of hemolytic streptococci or staphylococci showed no visible change in the organisms either in form, size, or staining properties. The pneumococci used as substrate in this experiment were of another type than those from which the enzyme was derived. This fact would indicate that the bacteriolytic enzyme is not type-specific, since it exerts a lytic action on strains of pneumococci serologically unrelated. The bacteriolytic effect of pneumococcus enzyme on *Streptococcus viridans*, although distinct, is much slower, being complete only after the action is continued for several hours. On the other hand, the strains of *Streptococcus hæmolyticus* and *Staphylococcus aureus* studied in the present investigation were uninfluenced by the action of the pneumococcus enzyme, and even after prolonged exposure showed no visible changes morphologically or tinctorially.

In controls, in which no enzyme had been added to the suspensions of heated bacteria, the organisms were still of normal appearance and gave no evidence of changes in coloring or form even after incubation for 20 hours at 37°C. Pneumococci acted upon by enzyme for this length of time appeared only as Gram-negative amorphous material; the green-producing streptococci were at this stage undergoing further disintegration, in stained films only an occasional Gram-positive form was seen, and the majority of the organisms appeared as faint Gram-negative shadows. Even after prolonged action of the pneumococcus enzyme on the substrate, the hemolytic streptococci and staphylococci were well preserved, retained their staining properties, and showed no evidence of bacteriolysis. After incubation for 48 hours, the tubes in which active bacteriolysis had occurred, namely those containing pneumococci, and streptococci of the *viridans* type, showed evidence of clearing with disappearance of the bacterial whirl, and were much more translucent than the control tubes to which no enzyme had been added. In the case of hemolytic streptococci and staphylococci no noticeable change in the gross appearance of the digestion mixture was evident.

From this experiment it appears that pneumococci possess an active intracellular agent which has the property of causing lysis of the bodies of heat-killed pneumococci, and, furthermore, that the lytic agent is not type-specific, since strains of pneumococci serologically different are equally affected. The bacteriolytic enzyme of pneumococcus also possesses to considerable degree the ability to attack streptococci of the *viridans* variety, cocci which biologically are more closely related to pneumococci than are hemolytic streptococci and staphylococci. Cocci of the last two varieties are apparently unaffected by the action of the enzyme, even after prolonged contact.

Influence of Hydrogen Ion Concentration on the Activity of the Intracellular Bacteriolytic Enzyme of Pneumococcus.

Under conditions similar to those described in Experiment 1, the effect of varying the hydrogen ion concentration on the bacteriolytic action of the enzyme was tested. In this experiment the enzyme solution was prepared from pneumococci of Type I and the bacterial substrate from pneumococci of Type II. The washed bacterial cells from 150 cc. of a plain broth culture of Pneumococcus Type II were suspended in 5 cc. of 0.1 M phosphate solution pH 7.4, and autoclaved at 15 pounds pressure for 20 minutes. After being killed by heat, the organisms were again centrifuged and the bacterial residue was washed and resuspended in 2 cc. of sterile distilled water. 0.1 cc. of active

enzyme solution and 0.1 cc. of bacterial substrate were now added to tubes each containing 1 cc. of 0.1 M phosphate solution. The tubes were adjusted to the desired hydrogen ion concentration by the addition, in the more acid ranges, of hydrochloric acid. The results of this experiment are shown in Table I.

TABLE I.

Influence of Hydrogen Ion Concentration on the Activity of the Bacteriolytic Enzyme of Pneumococcus.

pH	Enzyme solution of Pneumococcus Type I.	Substrate of heat-killed Pneumococcus Type II.	0.1 M PO_4 solution.	Lysis after 2 hrs. at 37°C.
	cc.	*cc.*	*cc.*	
2	0.1	0.1	0.8	−
	0	0.1	0.8	−
3	0.1	0.1	0.8	−
	0	0.1	0.8	−
4	0.1	0.1	0.8	−
	0	0.1	0.8	−
5	0.1	0.1	0.8	++
	0	0.1	0.8	−
6	0.1	0.1	0.8	+++
	0	0.1	0.8	−
7	0.1	0.1	0.8	+++
	0	0.1	0.8	−
7.8	0.1	0.1	0.8	+++
	0	0.1	0.8	−
8	0.1	0.1	0.8	+++
	0	0.1	0.8	−

+++ indicates complete lysis; ++, marked lysis; −, no lysis.

The lytic action of the enzyme was evident in the zone pH 5 to 8. At reactions more acid than pH 4 there was no evidence of lysis. At pH 3 a marked precipitation occurred. This may possibly be attributed to acid agglutination which for Type II pneumococci occurs at about this reaction. The optimum zone of hydrogen ion concentra-

tion, for the action of the bacteriolytic enzyme, corresponds closely to that already described for the peptonase, lipase, invertase, and inulase of pneumococcus.

Influence of Concentration of Enzyme on the Activity of the Intracellular Bacteriolytic Enzyme of Pneumococcus.

That the lytic action of solutions containing the intracellular substances of pneumococcus is enzymotic in nature, is evidenced by the following experiment in which it is shown that the rate and amount of lysis are a function of the concentration of the enzyme present. The conditions of this experiment were similar to those already described in previous experiments; the enzyme-containing solution was prepared

TABLE II.

Influence of Concentration of Enzyme on the Activity of the Bacteriolytic Enzyme of Pneumococcus.

Enzyme solution of Pneumococcus Type II.	Substrate of heat-killed Pneumococcus Type II.	0.1 M PO_4 solution pH 7.8.	Lysis after 1 hr. at 37°C.
cc.	*cc.*	*cc.*	
0.1	0.5	0.5	++++
0.05	0.5	0.5	+++
0.025	0.5	0.5	++
0.0125	0.5	0.5	+
0.006	0.5	0.5	+
0.003	0.5	0.5	+
.0	0.5	0.5	0

++++ indicates complete lysis; 0, no lysis.

from Pneumococcus Type II. This particular enzyme preparation had been kept in the ice box for 27 days. The bacterial substrate consisted of Type II pneumococci which had been prepared as already described.

From Table II it is evident that the action of the intracellular bacteriase is directly proportional to the concentration of the enzyme. It is interesting to observe that the activity of this enzyme, even after preservation in cold for 4 weeks, was still pronounced in quantities as small as 0.025 cc., and that traces of its action were detected in minimum amounts of 0.003 cc.

Influence of Heat on the Activity of the Intracellular Bacteriolytic Enzyme of Pneumococcus.

The following experiment was carried out to determine the effect of heat upon the bacteriolytic enzyme of pneumococcus.

300 cc. of plain broth pH 7.8 were seeded with a large inoculum (0.5 cc.) of a culture of Pneumococcus Type II. After 8 hours growth at 37°C., the culture was centrifuged and the bacteria were suspended in 20 cc. of 0.1 M phosphate solution at pH 7.2. To 10 cc. of this bacterial suspension, representing the cells from 150 cc. of culture, 0.2 cc. of undiluted ox bile was added. Solution of the organisms was marked after 30 minutes in the water bath at 37°C. and was complete after standing in the ice box over night. The remaining 10 cc. of the bacterial suspension to be used as substrate were autoclaved at 15 pounds pressure for 10 minutes in order to kill the organisms and to destroy the intracellular ferments.

TABLE III.

Influence of Heat on the Activity of the Bacteriolytic Enzyme of Pneumococcus.

Enzyme solution of Pneumococcus Type II, 0.5 cc.	Substrate of heat-killed Pneumococcus Type II.	Lysis.	
		After 2 hrs. at 37° C.	After 18 hrs. at 37° C.
	cc.		
Heated at 60°C. for 30 min.	0.5	−*	−
" " 80° " " 30 "	0.5	−	−
" " 100° " " 30 "	0.5	−	−
Unheated	0.5	++	++
No enzyme; phosphate solution and bile (1:50)†	0.5	−	−

* − indicates no lysis; ++, complete lysis.

† This shows that bile alone has no lytic effect on heat-killed pneumococci.

Aliquot portions of the solution containing the dissolved bacteria were heated in a water bath for 30 minutes at 60°, 80°, and 100°C., respectively. These heated solutions in 0.5 cc. amounts were now added to equal quantities of the suspension of heat-killed pneumococci. The results of this experiment are shown in Table III.

Analysis of Table III shows that exposure in the water bath to a temperature of 60°C. for 30 minutes destroys the activity of the bacteriolytic enzyme of pneumococcus. The resistance of the bacteriolytic enzyme of pneumococcus to heat is markedly less than that of the lytic agent of *Bacillus pyocyaneus* which, according to Emmerich (2), withstands steam at 100°C. for 2 hours.

DISCUSSION.

Evidence has been presented in this paper that pneumococci contain a bacteriolytic enzyme. This enzyme is associated with a number of other active intracellular agents which exert their effect upon various substances and which because of their enzymotic nature have been described as the intracellular peptonase, lipase, inulase, and invertase of pneumococcus. The bacteriolytic enzyme possesses the property of causing lysis of the dead bacterial bodies of pneumococci, and to a less extent the disintegration of closely allied organisms, such as *Streptococcus viridans*. However, it has no effect upon certain other Gram-positive cocci, as *Staphylococcus aureus* and *Streptococcus hæmolyticus*. The enzyme is not type-specific in its action, since an enzyme solution prepared from pneumococci of one type exerts a comparable action upon pneumococci of a heterologous type.

Emmerich, Löw, and Korschun (3) have demonstrated an enzyme in cultures of *Bacillus pyocyaneus* which possesses a remarkable lytic power. This enzyme, pyocyanase, in extraordinarily small amount, is capable of causing lysis of a number of other microorganisms such as *Bacillus diphtheriæ*, *Vibrio choleræ*, *Bacillus typhosus*, *Baciilus pestis*, streptococcus, and staphylococcus. In contrast with the pneumococcus enzyme, the enzyme from *Bacillus pyocyaneus* manifests its action on the living bacterial cell. Pyocyanase is remarkably heat-stable, resisting a temperature of 100°C. for 2 hours (2). This lytic agent is considered by Emmerich and his coworkers to be different from the peptonizing enzyme of *Bacillus pyocyaneus*. Löw and Kozai (4) have also demonstrated a bacteriolytic enzyme in cultures of *Bacillus prodigiosus*. Emmerich and Löw (5) include these bacteriolytic enzymes in the group of nucleases, which act on the nucleoprotein of the bacterial cell.

In the present instance, no assumption is made as to the identity of the pneumococcus bacteriolytic enzyme, since it is not known what particular constituent or constituents of the bacterial cell are acted upon. Whether lysis of pneumococci under these circumstances is the result of a single enzyme or the product of the interaction of more than one, and whether the enzyme or group of enzymes concerned in autolysis of pneumococci play any part in this form of lysis are questions at present undecided.

The fact that the pneumococcus bacteriolytic enzyme apparently has no effect on the living cell suggests that the living organism may possess a protective mechanism, possibly of the nature of antiferment. It may be only when this mechanism has been interfered with or destroyed that the bacterial cell is exposed to the action of its own enzymes.

SUMMARY.

1. Pneumococci possess an active intracellular enzyme which causes lysis of heat-killed pneumococci of the same and heterologous types and to a less degree of a closely related organism, *Streptococcus viridans*.

2. The optimum reaction for lysis lies between pH 6 and 8.

3. The bacteriolytic action is proportional to the concentration of the enzyme.

4. Heating the enzyme for 30 minutes at 60°C. destroys its activity.

5. The possible relation of the enzyme to autolysis is discussed.

BIBLIOGRAPHY.

1. Avery, O. T., and Cullen, G. E., *J. Exp. Med.*, 1920, xxxii, 547, 571, 583.
2. Emmerich, R., *Centr. Bakt., 1te Abt., Orig.*, 1902, xxxi, 585.
3. Emmerich, R., Löw, O., and Korschun, A., *Centr. Bakt., 1te Abt., Orig.*, 1902, xxxi, 1.
4. Löw, O., and Kozai, T., *Bull. Coll. Agric., Tokio*, v, quoted from Fuhrmann, P. F., Vorlesungen über Bakterienenzyme, Jena, 1907.
5. Emmerich, R., and Löw, O., *Z. Hyg. u. Infectionskrankh.*, 1901, xxxvi, 9.

STUDIES ON BACTERIAL NUTRITION.

IV. Effect of Plant Tissue upon Growth of Pneumococcus and Streptococcus.

By H. J. MORGAN, M.D., and O. T. AVERY, M.D.

(*From the Hospital of The Rockefeller Institute for Medical Research.*)

(Received for publication, April 12, 1923.)

Study of the growth requirements of *Bacillus influenzæ* (1–4) indicated that for the cultivation of this organism at least two substances must be present in the culture medium. Both of these substances are present in red blood corpuscles. One, a vitamine-like substance, can be extracted not only from red blood corpuscles but from yeast and vegetable cells as well, and is relatively thermolabile. This substance has been called the V factor. The other substance is also present in red blood corpuscles and in plant tissue, but it is thermostable and is effective in amounts so small as to suggest the possibility that it functions as a catalyst. It has been called the X factor. These observations on the growth of *Bacillus influenzæ* in the absence of blood have been extended (5). The organism can be readily grown in plain broth in which small pieces of sterile, unheated yellow or white turnip, carrot, beet, parnsip, or sweet potato have been placed. Knowledge of the growth-stimulating property of unheated plant tissue for organisms as sensitive to cultural conditions as the bacteria of the hemophilic group encouraged an extension of the study to other groups of bacteria. The present paper presents the results of a study of the effect of plant tissue upon the growth of certain Gram-positive cocci.

Material.

Media.—The plant tissues used in this study were white potato, sweet potato, carrot, white turnip, yellow turnip, parsnip, beet, and banana. Care was observed in selecting fresh young specimens. With the same technique described in a previous communication (4),

sterile pieces of these vegetables were placed in phosphate broth of pH 7.8. When air is not excluded from media prepared in this way oxidation of the plant tissue results in brown discoloration of the vegetable and of the supernatant broth. To prevent this the media were sealed with sterile vaseline. The media could then be stored in the ice box for many weeks without discoloration.

Organisms.—Three strains of pneumococci, three of hemolytic streptococci, and five of non-hemolytic streptococci were employed in these experiments.

Growth of Pneumococcus in Media Containing Sterile Plant Tissue.

The rate of growth of pneumococcus in broth is dependent upon several factors, the most important of which are (*a*) the growth activity of the bacteria used for seeding (6), (*b*) the size of the inocula, and (*c*) factors pertaining to the media such as its pH (7), its content in nutritive substances, etc. Depending upon the balance between these factors, growth as determined by the number of viable organisms present at different intervals during the life of the culture is divisible into four periods: (*a*) period of lag, during which the number of viable organisms in the culture decreases, remains constant, or slowly increases; (*b*) logarithmic period, in which the culture maintains its maximum rate of growth; (*c*) stationary period, in which the maximum rate of growth is not maintained, the organisms present, though viable, reproducing more slowly or not at all; and (*d*) period of decline, in which the number of viable organisms decreases rapidly, the pneumococcus culture eventually becoming sterile (6).

Effect of the Addition of Sterile Unheated Potato on the Growth of Pneumococcus.

With the above facts in mind, an experiment was planned to show what effect the addition of sterile unheated plant tissue to fluid media would have on the growth of pneumococci. The period of lag was first studied. Plain broth containing sterile unheated potato was used as the test medium. As a control, broth to which 1 per cent of dextrose had been added, was employed. Dextrose is readily fermented by pneumococcus, and is known to accelerate the growth of this organism.

Experiment 1.—Into a 300 cc. flask containing 150 cc. of phosphate meat infusion broth (pH 7.6) were placed four cylindrical pieces of unheated, sterile potato, each about 1.5 cm. long and 0.5 cm. in diameter. The flask was then incubated at 37°C. to insure sterility. A control flask, containing 150 cc. of broth of the same lot, to which 1 per cent dextrose had been added, was prepared. With the media at 37°C. each flask was seeded with 0.1 cc. of a 6 hour broth culture of Pneumococcus Type I. The culture used for seeding was actively growing, as indicated by the presence of long chains in the stained film. At frequent intervals over a period of 9 hours, fractions of both the dextrose broth and the potato broth cultures were removed and estimations made of the number of viable organisms present in each. In making these estimates the following method was employed. Several accurate dilutions of the culture were made. 0.5 cc. from each of two or more of these dilutions was added to a tube of 1 per cent dextrose agar and plates were poured. The necessary calculations for the different dilutions having been made, the average

TABLE I.

Acceleration of Growth of Pneumococcus in Broth Containing Sterile Unheated Potato.

Time after seeding.	Log of No. of colonies per 0.5 cc. of culture.	
	Plain broth + potato.	Plain broth + 1 per cent dextrose.
hrs.		
0	3.7	3.8
1	4.0	4.1
2	4.2	4.0
3	4.9	3.8
4	5.1	
5	5.8	4.0
6	6.3	4.0*
7	7.1	4.0*
9	7.9	4.0*

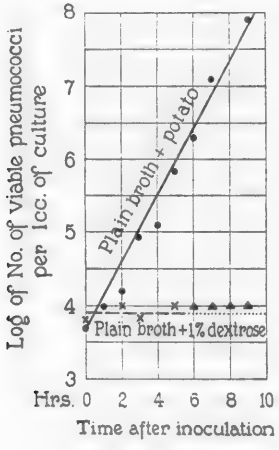
* No colonies in the dilutions plated. Therefore the exact determination was not made. The log is less than 4.

of the number of colonies present in the plates after 48 hours at 37°C. was used for the colony count per 0.5 cc. of culture for that hour. The results of these estimations as expressed in the logarithms of the number of colonies present at the time of each examination, are given in Table I. By using these logarithms as ordinates and the hours of examination as abscissæ, Text-fig. 1 was constructed.

From Table I and Text-fig. 1 it is evident that the organisms in broth containing sterile unheated potato grew steadily and at a constant rate. There occurred no period of lag. In contrast with this,

organisms seeded into broth containing an abundance of an easily fermentable substance, dextrose, but no plant tissue, failed to attain this maximum rate of growth for a period of at least 9 hours.

A second experiment was now performed in order to extend these observations and to determine the effect of the presence of unheated plant tissue upon the rate of growth during the entire cycle of a pneumococcus broth culture and also the effect upon changes in reaction which usually occur in the medium during growth. It is



TEXT-FIG. 1. Influence of sterile unheated plant tissue on the acceleration of growth of pneumococcus. ▲ indicates less than 4.

known that during growth of pneumococci in broth an increase in the pH occurs. When the medium is enriched by the addition of readily fermentable substances, as dextrose or certain other sugars, the acid production by the growing pneumococcus is still more marked (8).

Experiment 2.—The medium was prepared as in Experiment 1. Two 300 cc. flasks, one containing 150 cc. of 1 per cent dextrose broth, the other 150 cc. of plain broth to which had been added about 5 gm. of sterile unheated potato, were seeded with small identical amounts of a growing broth culture of Pneumococcus Type I. At intervals, as noted in Table II, samples were removed from each of the flasks. Estimations of the number of viable organisms were made, as in Experiment 1. Determinations of pH, by means of the colorimetric method, were made at the same time. The results of these observations are shown in Table II, and are graphically represented in Text-fig. 2.

From Table II and Text-fig. 2 it is seen that, under the conditions of the experiment, no period of lag occurred in the broth culture containing unheated potato. The control culture in dextrose broth, a medium quite favorable for prompt and abundant growth of pneumococcus when the inoculum is sufficiently large, showed a period of lag of 18 hours, during which the viable organisms in the culture apparently decreased in number. However, when once the culture

TABLE II.

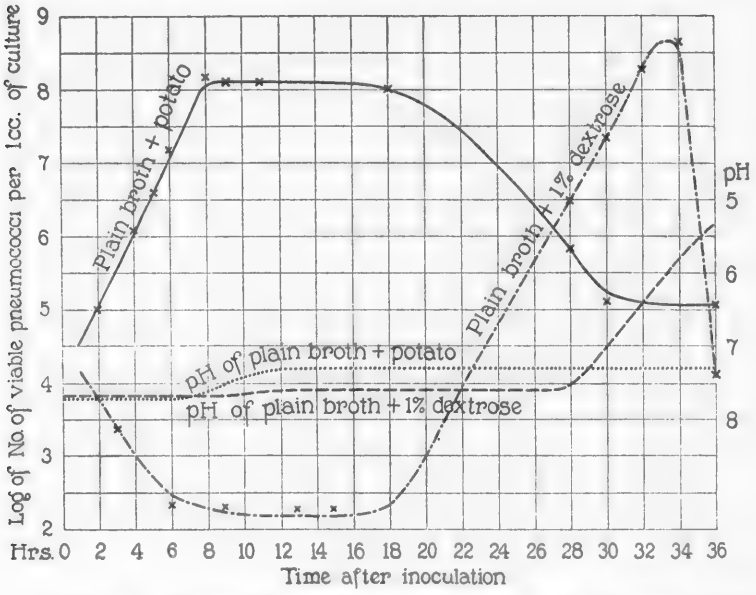
Comparison of Growth of Pneumococcus in Potato Broth and Dextrose Broth.

Time after seeding.	Broth containing unheated potato.		Broth containing 1 per cent dextrose.	
	Log of No. of colonies per 0.5 cc. of culture.	pH of culture.	Log of No. of colonies per 0.5 cc. of culture.	pH of culture.
hrs.				
1	4.5	7.7	4.2	7.7
2	5.0			
3	5.5		3.3	
4	6.1			
5	6.7	7.7		7.7
6	7.3		2.3	
8	8.2	7.5		7.65
9	8.1		2.3	
11	8.1			
13	8.1	7.3	2+*	7.6
15	8.1		2+*	
18	8.0		2.3	
28	5.8	7.3	6.5	7.6
30	5.1	7.3	7.4	7.1
32			8.4	6.3
34			8.7	5.3
36	5.1	7.3	4.1	

* Actual number of colonies not determined.

entered upon the logarithmic phase of growth, the generation time was approximately the same in the dextrose broth culture as in the potato broth culture; that is to say, cell division, when once initiated, proceeded at about the same rate in the two cultures. It is noteworthy that the stationary phase of the pneumococcus culture in potato broth extended over a period of 10 hours, while that of the culture in dextrose broth was of extremely short duration.

Other observations have shown that in the potato broth culture there is a prolongation of the period of decline in the growth curve; that is, the period in which cell death is proceeding at a more rapid rate than multiplication. It is especially prolonged when compared with a culture in dextrose broth. Moreover, when young actively growing potato broth cultures are removed from the incubator and are placed on ice, the bacteria may remain viable for very long periods



TEXT-FIG. 2. Influence of sterile unheated plant tissue on the growth curve of pneumococcus.

of time, even 8 months. These facts indicate that not only is the initiation of growth accelerated in the potato broth, but death of the bacteria occurs more slowly in this medium.

Effect of the Addition of Other Sterile Unheated Plant Tissues on the Growth of Pneumococcus.

The following experiment illustrates the effect on growth of pneumococcus when vegetable tissue, other than potato, is added to plain broth.

Experiment 3.—Sterile pieces of parsnip, sweet potato, carrot, yellow turnip, white turnip, and banana, each weighing about 0.5 gm., were placed in tubes containing 10 cc. of plain broth and these were sealed with vaseline. After incubating several days to insure sterility, the seals were removed and 2 cc. of the broth were taken from each tube for colorimetric pH determinations. Each tube was then seeded with 0.05 cc. of an actively growing broth culture of Pneumoccocus Type II. A tube containing 8 cc. of broth of the same lot, but no vegetable tissue, was seeded with the same amount of the same culture. The tubes were placed in the incubator at 37°C. Observations on the degree of clouding present in the media were made after 5 hours, and again after 17 hours incubation. The results are given in Table III.

TABLE III.

Acceleration of Growth of Pneumococcus in Broth Containing Sterile Unheated Vegetable Tissue.

Media.			Inoculum.	Growth of pneumococcus.*	
Broth.	Plant tissue.	pH at time of seeding.		5 hrs.	17 hrs.
cc.			*cc.*		
8	None.	7.8	0.05	−	++
8	Parsnip.	6.3	0.05	+	++++
8	Banana.	6.4	0.05	+++	++++
8	Sweet potato.	6.7	0.05	++	++++
8	Carrot.	6.9	0.05	++	++++
8	Yellow turnip.	7.1	0.05	++	++++
8	White "	7.2	0.05	±	++++
8	Potato.	7.4	0.05	+++	++++

* − indicates no macroscopic growth; ±, faint haze confined to vicinity of plant tissue; +, faint clouding throughout media; ++, moderate clouding throughout media; +++, marked clouding throughout media; ++++, maximum growth attained.

From Table III it is evident that other plant tissues have the same growth-stimulating effect upon pneumococcus that potato possesses. It is also evident that this stimulation operates in spite of the fact that the initial reaction of the media may be unfavorable for growth of this organism.

The Limiting pH for the Growth of Pneumococcus in Potato Broth.—It has been shown that the optimum hydrogen ion concentration for growth of pneumococcus is pH 7.8 and that growth in plain broth

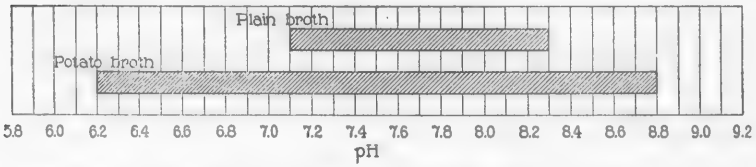
can be initiated only if the hydrogen ion concentration is between pH 7 and 8.3 (7). The observations made in Experiment 3 indicate that if there is present in the medium a bit of unheated vegetable tissue growth of pneumococcus can be initiated even though the hydrogen ion concentration is only pH 6.3. An attempt was made, therefore, to determine the limiting hydrogen ion concentrations for the initiation of growth of pneumococcus in broth containing sterile unheated potato.

TABLE IV.

Growth of Pneumococcus at Different Hydrogen Ion Concentrations in Broth Containing Sterile Unheated Plant Tissue.

Inoculum.	pH of media.	Growth of pneumococcus in plain broth after 18 hrs.*	Growth of pneumococcus in plain broth + potato after 18 hrs.*
cc.			
0.05	5.8	−	−
0.05	6.2	−	+
0.05	6.8	−	+
0.05	7.1	+	+
0.05	8.3	+	+
0.05	8.6	−	+
0.05	8.8	−	+
0.05	9.0	−	−

* − indicates no macroscopic growth; +, growth.



TEXT-FIG. 3. Influence of sterile unheated plant tissue on the range of hydrogen ion concentration for initiation of growth of pneumococcus.

Experiment 4.—Tubes containing 10 cc. of potato broth were prepared in the usual way and incubated to insure sterility. In a number of tubes the hydrogen ion concentration was adjusted, by the addition of suitable amounts of sterile N and 0.1 N HCl or NaOH, so that a series of tubes was obtained in which the reaction ranged between pH 5.8 and 9. In the same way there was prepared a series of tubes of plain broth, without potato, in which the hydrogen ion concentration ranged between the same points. All these tubes were then seeded, each with

0.05 cc. of a growing culture of Pneumococcus Type I, and incubated at 37°C.[1] Growth of pneumococcus occurred as is recorded in Table IV, and as represented diagrammatically in Text-fig. 3.

The results in Table IV indicate that the presence of sterile unheated potato in broth greatly increases the range of pH in which the growth of pneumococcus can be initiated. This increased range extends between pH 6.2 and 8.8.

Effect of the Addition of Sterile Unheated Potato on the Growth of Hemolytic and Non-Hemolytic Streptococci.

In order to determine whether unheated plant tissue might exert a growth-stimulating effect on other cocci, strains of hemolytic and of non-hemolytic streptococci were grown in plain broth alone and in the same broth to which unheated plant tissue had been added. Differences in growth activity of the same organisms in the two types of media were noted.

TABLE V.

Acceleration of Growth of Hemolytic and Non-Hemolytic Streptococci in Broth Containing Sterile Unheated Plant Tissue.

Organisms.	Growth in 10 cc. of plain broth.*	Growth in 10 cc. of potato broth.*
Results after 14 hrs. incubation.		
1. Hemolytic Streptococcus 3/22	−	+++
2. " " 23/20	−	+++
3. " " 84/18	−	+++
Results after 18 hrs. incubation.		
4. Non-hemolytic Streptococcus A141	−	++
5. " " A135	−	+
6. " " B39	+	+++
7. " " 38d	±	+++
8. " " A149	±	++

* − indicates no macroscopic growth; ±, faint haze; +, bacterial whirl; ++, marked growth; +++, maximum growth.

[1] A set of uninoculated tubes containing potato broth at reactions between pH 7.2 and 4.6 was also incubated to determine whether plant tissue alone, at 37°C., might cause a change in the pH. After 3 days at 37°C. the pH determinations on the broth in these tubes showed that no changes had occurred.

Experiment 5.—The media were incubated for 3 days to insure sterility. 24 hour blood broth cultures of three strains of hemolytic streptococci and five strains of non-hemolytic streptococci were used for seeding. In each instance the tip of a platinum wire was inserted the distance of 1 cm. into the supernatant fluid of the blood broth culture and then carried into the test medium. The latter was then placed in the incubator at 37°C. The degree of clouding of the medium was noted at the hours given in Table V.

From the results shown in Table V it is evident that sterile unheated plant tissue has a marked growth-stimulating effect upon hemolytic and non-hemolytic streptococci. Heavy clouding of the potato broth cultures of the three strains of *Streptococcus hæmolyticus* and, to a less degree, of two of the five strains of non-hemolyzing streptococci occurred before there was macroscopic evidence of growth of these organisms in the plain broth without potato. In the remaining cultures of non-hemolytic streptococci at the end of 18 hours growth was much more abundant in the potato broth tubes than in the tubes of plain broth.

SUMMARY.

In previous papers it has been shown that unheated plant tissue, in the form of potato, contains the two factors necessary for the growth of organisms of the hemoglobinophilic group. Further studies (5) confirmed these findings and showed that yellow and white turnip, carrot, beet, parsnip, and sweet potato can replace blood in the cultivation of *Bacillus influenzæ*.

In the present paper it has been shown that vegetable tissues also greatly facilitate and stimulate the growth of other organisms entirely unrelated to *Bacillus influenzæ*. Three varieties of Gram-positive cocci have been used in the present study, pneumococcus, *Streptococcus hæmolyticus*, and *Streptococcus viridans*. With pneumococcus it has been previously shown that prompt and luxuriant growth will occur in broth containing unheated potato even though the seeding be so small that no growth whatever will occur with the same seeding in plain broth (5). In the present study it has been shown that even in dextrose broth this minimal inoculation is followed by a prolonged period of lag, whereas in potato broth this same inoculum serves to initiate immediate and rapid growth. When pneumococci are grown in potato broth not only is the period of lag abolished, but the sta-

tionary period of growth is extended and cell death is delayed. Moreover, in plant tissue medium the zone of hydrogen ion concentration within which growth of pneumococcus can be initiated is considerably extended beyond the acid and alkaline limits of the optimal range in ordinary bouillon.

It has been found also that the presence of unheated plant tissue in the media likewise stimulates growth of hemolytic and non-hemolytic streptococci.

In this investigation no attempt has been made to determine the exact nature of the substances in plant tissue upon which these properties depend. That they are not of the nature of readily fermentable carbohydrates, however, is made evident by the fact that no increased production of acid occurs in the pneumococcus culture when potato is present.

CONCLUSIONS.

1. The initiation of active growth in broth culture of pneumococcus, *Streptococcus hæmolyticus*, and *Streptococcus viridans* is accelerated by the presence of unheated plant tissue.

2. Cultures of pneumococcus in broth containing unheated plant tissue show a prolongation of the stationary phase of growth.

3. Death of the individual organisms in pneumococcus broth cultures containing unheated plant tissue does not proceed so rapidly as in cultures without plant tissue.

4. The zone of hydrogen ion concentration within which growth of pneumococcus in ordinary broth can be initiated is considerably extended, both on the acid and on the alkaline side, by the addition of unheated vegetable tissue to the media.

BIBLIOGRAPHY.

1. Thjötta, T., *J. Exp. Med.*, 1921, xxxiii, 763.
2. Thjötta, T., and Avery, O. T., *Proc. Soc. Exp. Biol. and Med.*, 1920–21, xviii, 197.
3. Thjötta, T., and Avery, O. T., *J. Exp. Med.*, 1921, xxxiv, 97.
4. Thjötta, T., and Avery, O. T., *J. Exp. Med.*, 1921, xxxiv, 455.
5. Avery, O. T., and Morgan, H. J., *Proc. Soc. Exp. Biol. and Med.*, 1921–22, xix, 113.
6. Chesney, A. M., *J. Exp. Med.*, 1916, xxiv, 387.
7. Dernby, K. G., and Avery, O. T., *J. Exp. Med.*, 1918, xxviii, 345.
8. Avery, O. T., and Cullen, G. E., *J. Exp. Med.*, 1919, xxx, 359.

THE COMPLEMENT FIXATION REACTION AS APPLIED TO LEPROSY.

By PAUL A. LEWIS, M.D., and JOSEPH D. ARONSON, M.D.

(*From the Henry Phipps Institute of the University of Pennsylvania, Philadelphia.*)

(Received for publication, February 27, 1923.)

A survey of the more recent literature relative to leprosy shows that many have sought to use the reaction of complement fixation as a means of increasing our knowledge of this disease from one or another of several points of view. Some have hoped to increase our ability to make a certain diagnosis, using the serum of the supposed leper against an extract or suspension of materials derived from the nodular lesions characterizing the disease, employed as an antigen. Others have hoped that by considering that the serum of the leper contained a specific antibody, the use of extracts or suspensions of various bacterial cultures derived from leprous lesions might enable them to assign to certain of these, antigenic qualities, which would help to determine an etiological relationship to the disease. As a development related to the latter purpose, some have immunized animals with certain cultures and sought to determine by agglutination or complement fixation their relation to other bacteria, particularly those of the acid-fast group. In no instance have these efforts to apply complement fixation been conspicuously successful, nor is there so far apparent in the literature any satisfactory generalization covering the work which has been done. The results often seem contradictory and many of them are not even understandable so long as one confines himself to the printed reports.

Our previous work on complement fixation in tuberculosis (55, 57) had led us to a different point of view with regard to this reaction as applied to bacterial antigens, particularly those of the acid-fast group. It appears that the method of Bordet and Gengou, potentially a very general one and susceptible in theory to much modification, has in practise become conventionalized and confined within bounds determined by the marked success of a single application; namely, the Wassermann reaction as related to syphilis. Most who have sought to apply

the complement fixation reaction to the study of either tuberculosis or leprosy have followed rather precisely one or another of the modifications of the Wassermann reaction.

We found, as a matter of fact, that the fixation reaction with the suspensions of acid-fast bacteria or their extracts ran at a slower rate than the reaction between the active principle of the syphilitic serum and the lipoidal extracts commonly used as Wassermann antigens. Whereas these give perfect fixation in from 20 minutes to 1 hour at 37°C., the reaction with acid-fast bacterial suspensions or extracts from them is scarcely evident in 1 hour and is not complete for 2, or even in certain instances for 4 hours. The intricacies of the technique are such that confusing and apparently positive results may frequently appear within the hour, but when the foregoing point had been developed it was clear that these were accidental. Our observations with tuberculosis are here applied to the study of the serum of a number of cases of leprosy[1] and suitable control sera.

The literature of the subject is extensive and, as has been indicated, not conclusive in its bearings. Since our method is novel we do not devote any considerable space to a formal discussion of the work of our predecessors. A bibliographic index is appended for the use of anyone who may be interested in a review. Cooke in his first paper (8) gives a tabulation of the results obtained up to 1919 from the diagnostic point of view. It is evident from his summary that leprosy cases do give complement fixation in varying degree with a number of antigens, and there is a clear indication that the reaction is in large measure non-specific. So it is now commonly stated that many lepers give positive Wassermann reactions and likewise react with the products of the tubercle bacillus as antigens. This has been held to make the results impossible of interpretation in the diagnostic sense, a point which will be discussed below. It is especially worthy of note that while Cooke was able to tabulate upward of 2,000 cases of leprosy whose blood had been thus examined up to 1919, he does not tabulate the results with non-lepers, and as a matter of fact our study of the literature has shown that few such control

[1] For the serum from leprosy cases we are much indebted to Dr. O. E. Denney, of the United States Public Health Service Hospital No. 66, at Carville, La., and to Dr. G. W. McCoy, Director of the Hygienic Laboratory of that service, who introduced us to Dr. Denney.

tests have ever been reported. Of the controls reported a number have been syphilitic sera, and these have often given positive fixations with leprosy antigens. This we believe due to inherent defects in the methods employed and we have not, therefore, collected the results or laid stress on them in this discussion.

During the course of our studies with tuberculosis we have developed an immune serum of high potency by treating a goat with living culture of an avirulent bovine type of tubercle bacillus. We sought by experiment to determine the degree of specificity which might pertain to this serum. It was found that as between the bovine and human type of tubercle bacillus no differences could be made out. The avian type gave distinctly less reaction when used as antigen. Other cultures in the acid-fast group commonly carried in stock laboratories as saprophytic acid-fast bacteria gave no reaction whatever with this serum.

The cultures which have been associated with leprosy by reason of their origin reacted variously.[2] *Bacillus lepræ* (Duval) and *Bacillus lepræ* (Kedrowsky) have given, with this goat serum, reactions of about the same quantitative value as the avian tubercle bacillus. *Bacillus lepræ* (Clegg) gives no reaction whatever. In extension of this it was found that when human serum from cases of tuberculosis was used they might react somewhat with Duval, Kedrowsky, or avian tubercle cultures but never with Clegg. It did seem possible then, in view of the clear-cut results which the complement fixation reaction as used by us was capable of giving, that an examination of lepra serum might develop points of interest.

Methods.

Antigens.—A number of antigens were employed.

(*a*) *Bacillary Emulsions.*—These emulsions were prepared by removing the organisms from the slant of glycerol agar, weighing the mass, and adding sufficient salt solution to bring about a concentration of 10 mg. per cc. In order to make

[2] These cultures have come to us from Professor C. W. Duval. They are: (1) A culture of the type isolated by Clegg, which tends to grow in a heavy moist mass and produces much light yellow pigment. The culture which we have used was isolated by Duval but is spoken of throughout as *B. lepræ* (Clegg). (2) A culture isolated by Duval which originally was propagated with great difficulty and which produces no pigment. This is spoken of as *B. lepræ* (Duval). (3) A culture of similar characters isolated by Kedrowsky and spoken of by his name.

this emulsion as uniform as possible, the organisms were first ground in an agate mortar and the salt solution added slowly. The emulsion was then filtered through cotton in order to remove the larger clumps which might remain. Emulsions were prepared from the following organisms: *B. lepræ* (Clegg), *B. lepræ* (Duval), *B. lepræ* (Kedrowsky), *B. smegmatis*, Timothy hay bacillus, Mist bacillus, Marpman's urine bacillus, Rabinowitch's bacillus, butter bacillus, an acid-fast bacillus which has been designated as paratuberculosis, and a saprophytic chromogenic acid-fast organism which we have isolated and designated "Jacob."[3]

(*b*) *Alcoholic Extracts.*—An alcoholic extract of the bovine *B. tuberculosis* and of *B. lepræ* (Clegg) was prepared by first growing the organisms on glycerol bouillon until the surface of the liquid was covered. The culture was then filtered through filter paper and the mass of bacteria washed several times with salt solution until the filtrate was clear. The bacterial mass was collected and placed in a vacuum desiccator over sulfuric acid until dry. The dried organisms were placed in a Soxhlet extractor, and extraction with boiling absolute ethyl alcohol was continued for a week or longer, the alcohol being renewed several times during the period of extraction. The extracts, before being used, were brought to the boiling point, and a measured amount of the hot extract was added to cold salt solution to make the desired concentration.

(*c*) *Petroff's Antigen.*—A glycerol extract of B. tuberculosis, prepared according to the method of Petroff (54) was employed as a control for the alcoholic extract of *B. tuberculosis.*

(*d*) *Cholesterol and Acetone Antigens.*—A cholesterolized alcoholic extract of human heart and an acetone-insoluble fraction prepared from the same organ were used as in the Wassermann reaction.

With the exception of the alcoholic extracts and Petroff's antigen, the recently prepared antigens were used, since we have found that the bacillary emulsions, even when kept at a low temperature, are not stable and in a short time become highly anticomplementary. The emulsions were diluted further with salt solution so that each cubic centimeter contained 1 mg. of the bacteria. One-sixth of the anticomplementary dose was employed in the reaction. This amount, varying from 0.1 to 0.2 mg., was found to be fairly constant with the organisms which give a homogeneous emulsion, while, with those cultures which did not give a homogeneous emulsion, the amount fluctuated more widely. The antigenic dose of the alcoholic extract of *B. tuberculosis* and of Petroff's antigen was determined by adding varying amounts of the diluted extracts to 10 units of the homologous immune goat serum, and the minimal fixing dose was ascertained. The alcoholic extract of *B. lepræ* (Clegg) was employed in one-sixth of the anticomplementary dose.

---

[3] The cultures of *B. smegmatis*, Timothy hay bacillus, Marpman's bacillus, Rabinowitch's bacillus, butter bacillus, Mist bacillus, and paratuberculosis bacillus were obtained from Dr. D. H. Bergey, of the Department of Bacteriology of the University of Pennsylvania.

Serum.—The leprosy blood was collected by Dr. Denney in Keidel tubes and mailed to us by parcel post. It was always in apparently good condition on arrival and was used as soon as possible. The serum separated clear and there is no definite reason to assume that it was seriously altered in any way. It should, however, be noted that the number of anticomplementary sera encountered was greater than with the control sera collected from non-lepers in Philadelphia, and this may be accounted for by the necessary delay in transport. The serum was inactivated at 56°C. for 30 minutes before use. 0.1 cc. of serum was used in the tests and 0.2 cc. as the control for anticomplementary action.

Complement.—The complement was obtained by bleeding guinea pigs from the heart. The blood was defibrinated and permitted to remain in contact with the clot over night. Blood was always obtained from two or more animals which had been previously tested to be sure that their complement was capable of giving fixation with tuberculosis antigens. The complement was separated by centrifuging and the unit of complement determined by mixing varying amounts of complement with 2 units of amboceptor and 0.1 cc. of 5 per cent sheep cell suspension. After the addition of sufficient salt solution to bring the total to 1 cc., the tubes were placed in the incubator, and the minimum amount of complement which had completely hemolyzed the cells was taken as the unit.

Tests.—0.1 cc. of the serum was apportioned out in a number of tubes, to which was then added the antigen. 2 units of complement were added and then sufficient salt solution to bring the total volume to 1 cc. The tubes were incubated for 2 hours at 37°C., at the expiration of which time 4 units of anti-sheep amboceptor and 0.1 cc. of a 5 per cent suspension of sheep cells were added. After 30 minutes incubation in the water bath, the results were noted. The tubes were placed in the ice box and again read the next morning. The increase of the period of primary incubation to 2 hours is, we believe, one of the fundamental features of our method. The other feature of importance is the use of 4 or more units of anti-sheep amboceptor. The effect of this is to mask any variations due to the presence of natural anti-sheep amboceptor which occurs in variable amounts in human serum and possibly also to detach any loosely bound complement. In practise this procedure somewhat reduces the delicacy of the test but greatly increases the certainty of result. For a more complete discussion of this the reader is referred to previous papers on tuberculosis (55, 57).

EXPERIMENTAL.

Of the forty-five specimens of sera obtained from thirty-nine cases of leprosy, four were anticomplementary in a dose of 0.1 cc. but smaller quantities, except with one specimen, were not anticomplementary. A comparison of the anticomplementary property of non-leprous sera with that of leprous sera shows that, while the non-leprous were anti-

complementary in 2.3 per cent of 604 sera examined, sera from leprosy cases were anticomplementary in 8.8 per cent of forty-five cases studied, when the same dose of serum was employed.

An attempt was made to determine, by means of the method outlined above, in what way non-leprous sera differed in their complement-fixing property from leprous sera.[4] The results of the complement fixation reaction with leprous sera and non-leprous sera and with various antigens are shown in comparison in Table I.

It will be noted that the leprosy sera give reactions quite generally with all of the antigens employed. While the differences are not great, the number of reactions with the tubercle bacillus or its products is ac-

TABLE I.

Antigen.	Leprous serum.				Non-leprous serum.			
	No.		Per cent.		No.		Per cent.	
	Positive.	Negative.	Positive.	Negative.	Positive.	Negative.	Positive.	Negative.
B. lepræ (Clegg) alcoholic extract	41	3	93.2	6.8	0	152	0.0	100.0
" " "	31	2	93.9	6.1	0	120	0.0	100.0
" " (Duval)	36	8	81.8	18.2	51	99	34.0	66.0
" " (Kedrowsky)	37	6	86.1	13.9	35	56	38.5	61.5
" *tuberculosis*, alcoholic extract	43	1	97.7	2.3	48	104	31.6	68.4
Petroff's antigen (T.B.)	30	1	96.8	7.2	11	28	28.2	71.8
Cholesterol (Wassermann reagents)	29	15	65.9	34.1	37	115	24.3	75.7
Acetone-insoluble (Wassermann reagents)	28	16	63.6	36.4	25	109	18.7	81.3

tually higher than with any of the cultures having leprosy as a source. The reaction is, then, as has generally been assumed, non-specific. The result can have little value in terms of the etiological significance of any of the cultures derived from lepers. On the other hand, certain of the leprosy cultures do give a high percentage of reaction with the control sera, which may possibly indicate a close relationship of the cultures Duval and Kedrowsky with the tubercle bacillus.

[4] For this purpose sera were employed which were obtained from patients who attended the prenatal clinic, the clinic for the diseases of the chest, and the clinic for syphilitics at the Henry Phipps Institute, Philadelphia. Many of these cases are known to have tuberculosis or syphilis or both diseases.

Among the control sera none were found to give a reaction with *Bacillus lepræ* (Clegg). This observation has been amplified in two directions. First, an attempt was made to determine whether the reaction with *Bacillus lepræ* (Clegg) might be specific if examined quantita-

TABLE II.

Case No.	Type.	Least quantity of serum reacting with antigens given.				
		B. lepræ, chromogenic.	*B. lepræ*, non-chromogenic.	*B. lepræ* (Kedrowsky).	*B. tuberculosis.*	Cholesterol.
		cc.	*cc.*	*cc.*	*cc.*	*cc.*
108	Anesthetic.	Negative.	Negative.	Negative.	0.05	Negative.
112	Nodular.	0.01	0.02	0.02	0.02	0.02
151	Anesthetic.	Negative.	Negative.	Negative.	0.05	Negative.
203	Nodular.	0.02	0.02	0.05	0.02	"
210	Mixed.	0.01	0.01	0.02	0.02	0.01
211	"	0.01	0.02	0.02	0.02	0.05
187	Anesthetic.	0.01	0.05	0.02	0.01	0.01
172	Nodular.	0.02	0.1	0.05	0.01	0.1
197	"	0.01	0.05	0.05	0.02	0.05
209	"	0.01	0.05	0.05	0.02	0.05
15	Mixed.	0.01	0.01	0.01	0.01	0.02
25	Nodular.	0.01	0.01	0.01	0.01	0.1
95	"	0.01	0.01	0.01	0.01	0.1
134	Mixed.	0.01	0.01	0.01	0.01	0.02
151	Anesthetic.	0.10	0.01	0.01	0.02	Negative.
83	Mixed.	0.01	0.01	0.01	0.02	"
174	"	0.01	0.01	0.01	0.01	0.01
217	Anesthetic.	0.02	0.01	0.01	0.02	Negative.
5	Nodular.	0.01	0.01	0.01	0.01	0.02
157	Mixed.	0.01	0.01	0.01	0.01	0.01
186	Anesthetic.	0.02	0.01	0.01	0.02	Negative.
173	Mixed.	0.01	0.01	0.01	0.01	0.01
144	Nodular.	0.01	0.01	0.01	0.01	0.05
203	"	0.01	0.01	0.01	0.01	0.1
150	Mixed.	0.02	0.01	0.02	0.05	0.1
177	"	0.01	0.01	0.01	0.01	0.01

tively. The differences in this sense as shown in Table II are not material.

Secondly, the study was extended to include a considerable number of antigens prepared with other acid-fast bacteria. Again it is seen in Table III that the reaction of leper serum with this group of antigens is

more or less universal. It is true that with none of the saprophytic acid-fast bacteria is the percentage of reacting serum so high as with *Bacillus lepræ* (Clegg), and this applies also to *Bacillus lepræ* (Kedrowsky) and *Bacillus lepræ* (Duval). On the other hand, among the control sera there is an almost universal failure to react with the cultures to which no suspicion of pathogenicity attaches. Two sera reacted with "Jacob," one of these also with syphilitic reagents, the other also with *Bacillus lepræ* (Duval) and *Bacillus lepræ* (Kedrowsky).

TABLE III.

Antigen.	Leprous serum.				Non-leprous serum.			
	No.		Per cent.		No.		Per cent.	
	Positive.	Negative.	Positive.	Negative.	Positive.	Negative.	Positive.	Negative.
B. lepræ, chromogenic, alcoholic extract	14	1	93.3	6.7	0	33	0.0	100.0
" " " (Clegg)	13	2	86.7	13.3	0	33	0.0	100.0
" " non-chromogenic (Duval)	9	6	60.0	40.0	6	26	18.7	81.3
" " (Kedrowsky)	11	4	73.3	26.7	7	26	21.2	78.8
" *tuberculosis*, alcoholic extract	14	1	93.3	6.7	8	27	22.9	77.1
Cholesterol	9	6	60.0	40.0	8	27	22.9	77.1
Acetone	8	7	53.3	46.7	5	30	14.3	85.7
Timothy hay bacillus	12	3	80.0	20.0	0	30	0.0	100.0
Marpman's urine bacillus	4	11	26.7	73.3	0	31	0.0	100.0
Rabinowitch's bacillus	6	9	60.0	90.0	0	29	0.0	100.0
Butter bacillus	10	5	66.7	33.3	0	28	0.0	100.0
Mist bacillus	9	6	60.0	40.0	0	27	0.0	100.0
Paratuberculosis bacillus	9	6	60.0	40.0	0	25	0.0	100.0
"Jacob"	11	4	73.3	26.7	2	23	8.0	92.0
B. smegmatis	7	8	46.7	53.3	0	22	0.0	100.0

It is not altogether clear why, considering for the moment that *Bacillus lepræ* (Clegg) may be saprophytic and incidental in its relation to the lesions of leprosy, it should still be a better antigen than other acid-fast bacteria. It makes a better emulsion than many of them, which may have an influence, but this is for the present only a suggestion. If it were possible for us to continue this work we should proceed to the examination of the alcoholic extracts of a number of other cultures, particularly *Bacillus lepræ* (Duval) and *Bacillus smegmatis*.

A classification of the results with the leper sera based on the type of disease is also presented (Table IV). That there exists a relationship between the type of the disease and the activity of the serum in the reaction of complement fixation has been recognized by a number of observers. From this table it is apparent that the mixed type of the disease yields the highest percentage of positively reacting sera while

TABLE IV.

Reactions According to Type of Leprosy.

Antigen.	Anesthetic.				Nodular.				Mixed.			
	Positive.		Negative.		Positive.		Negative.		Positive.		Negative.	
	No.	Per cent.	No.	Per cent.	No.	Per cent.	No.	Per cent.	No.	Per cent.	No.	Per cent.
B. lepræ, chromogenic	6	13.6	2	4.5	15	34.1	1	2.3	20	45.5	0	0.0
" " non-chromogenic	5	11.3	3	6.8	14	31.8	2	4.6	17	38.7	3	6.8
" " (Kedrowsky)	5	11.6	3	7.0	14	32.6	1	2.3	18	41.9	2	4.6
" *tuberculosis*, alcoholic extract	8	18.2	0	0.0	16	36.3	0	0.0	19	43.2	1	2.3
Petroff's antigen	5	16.7	0	0.0	8	26.6	0	0.0	16	53.4	1	3.3
Cholesterol	3	6.9	5	11.3	11	25.0	5	11.3	15	34.2	5	11.3
Acetone	3	6.9	5	11.3	11	25.0	5	11.3	14	31.9	6	13.6
Timothy hay bacillus	2	13.3	0	0.0	3	20.0	1	6.7	7	46.7	2	13.3
Marpman's bacillus	0	0.0	2	13.3	1	6.7	3	20.0	3	20.0	6	40.0
Rabinowitch's bacillus	2	13.3	0	0.0	1	6.7	3	20.0	3	20.0	6	40.0
Butter bacillus	2	13.3	0	0 0	2	13.3	2	13.3	6	40.0	3	20.0
Mist bacillus	2	13.3	0	0.0	2	13.3	2	13.3	5	33.4	4	26.7
Paratuberculosis bacillus	2	13.3	0	0.0	2	13.3	2	13.3	5	33.4	4	26.7
"Jacob"	2	13.3	0	0.0	3	20.0	1	6.7	6	40.0	3	20.0
B. smegmatis	1	6.7	1	6.7	2	13.3	2	13.3	4	26.6	5	33.4

the anesthetic type yields the smallest number of such sera. There does not appear to exist any relationship between the type of disease and any given antigen.

Reasoning from analogy it is conceivable that absorption experiments with the acid-fast bacterial antigens might lead to some differentiation in instances in which the direct analysis has given none. Such experiments have failed in the past because when serum is incubated with suspensions of acid-fast bacteria and the latter are removed by centrifugalization the resulting fluid is anticomplementary. It was found by one of us that this difficulty could be avoided by removing the bacterial

bodies by filtration through Berkefeld or Mandler candles. The complete removal of the complement-fixing bodies from a strong antituberculosis serum may be satisfactorily demonstrated in this way. We have sought to apply this observation to the present problem. The complement-fixing body of leper serum is not removed by absorption either with *Bacillus tuberculosis* or with *Bacillus lepræ* (Clegg). Neither is it removed by treatment with charcoal or kaolin, as are many antibodies.

DISCUSSION.

The wide range of antigenic substances fixed by serum from cases of leprosy precludes the use of the complement fixation reaction for the determination of the specific organism in the causative sense. Absorption with the bacterial suspensions does not remove the complement-fixing bodies from the serum.

The fact that leprosy sera give so high a percentage of positive reactions with antigens ordinarily used for the Wassermann reaction and with the tuberculosis antigens cannot be interpreted as being indicative of a superimposed or underlying infection with either syphilis or tuberculosis. When one realizes that only 8 to 10 per cent of the general population give a positive Wassermann reaction and that extremely unmoral groups give about 30 per cent, it does not seem reasonable to suppose that 66 per cent of lepers are syphilitic. As further suggestion that a positive Wassermann reaction in leprosy is not necessarily indicative of an accompanying syphilitic infection, is the fact that the Wassermann reaction in leprosy is not affected by the administration of arsenicals. Again, in earlier studies in complement fixation (55, 57) with the same antigens that were employed in this study, it was found that but 71 per cent of cases of frank tuberculosis gave a positive complement fixation reaction with tuberculosis antigen, whereas with the sera from leprosy cases a positive reaction was obtained with over 93 per cent of the cases, a higher percentage than with any of the other antigens employed.

We know of no other disease in which the blood serum develops this property of multiple fixation. If our controls are adequate this property might be of diagnostic value in either the positive or negative sense. However, opinion as to this point must for the present be reserved. The control sera which have been employed come from a different commun-

ity, and unforeseen complications to any interpretation of this order may conceivably arise. Granted that the results obtained are truly applicable, the fixation reaction done by our method with the alcoholic extract of *Bacillus lepræ* (Clegg), or with the emulsion of this organism, would seem likely to be of use practically in spite of the fact that it would at present have no clear foundation in any etiological relationship between this bacterium and leprosy as a disease.

SUMMARY.

By means of a method differing in important details from those of previous investigators it has been determined that the blood serum of cases of leprosy exhibits the ability to fix complement with a wide variety of antigens including to a greater or less extent those derived from any culture of the acid-fast group of bacteria available to us.

This property of multiple fixation may sufficiently characterize the disease to be of diagnostic significance, although our experience is hardly sufficient to enable us to speak with complete assurance on this point. Certainly, control sera from normal individuals, from cases of tuberculosis, or from cases of syphilis as obtained in our locality have entirely failed to react with certain antigens, whereas serum from cases of leprosy have so reacted to the extent of over 93 per cent.

The most characteristic fixation given by the leprosy sera is that with *Bacillus lepræ* (Clegg) used as antigen, either in the form of a bacterial emulsion or of an alcoholic extract of the dried culture.

Antibody absorption may be demonstrated in the acid-fast group if the absorbing bacteria are removed by filtration. Otherwise the resulting fluid is strongly anticomplementary. Leper serum is not deprived of the complement-fixing body when so treated with either *Bacillus tuberculosis* or *Bacillus lepræ* (Clegg).

BIBLIOGRAPHY.

The following bibliography has been arranged on the basis of the nature of the antigen discussed in the publication.

(*a*) *Extract of Lepromas.*

1. Eitner, E., *Wien. klin. Woch.*, 1906, xix, 1555.
2. Eitner, E., *Wien. klin. Woch.*, 1908, xxi, 729.
3. Frugoni, C., and Pisani, S., *Berl. klin. Woch.*, 1909, xlvi, 1530.

4. Thomsen, O., and Bjarnhjedinson, S., *Lepra*, 1909–10, ix, 191.
5. Nishiura, K., *Z. Immunitätsforsch., Orig.*, 1910, vii, 721.
6. Akerberg, H., Almkvist, J., and Jundell, I., *Lepra*, 1910, ix, 79.
7. Steffenhagen, K., *Berl. klin. Woch.*, 1910, xlvii, 1362.
8. Cooke, J. V., *J. Infect. Dis.*, 1919, xxv, 474.
9. Gaucher and Abrami, P., *Lepra*, 1909, viii, 152.
10. Slatinéanu, A., and Daniélopolu, D., *Compt. rend. Soc. biol.*, 1908, lxv, 309.
11. Slatinéanu, A., and Daniélopolu, D., *Compt. rend. Soc. biol.*, 1908, lxv, 702.
12. Serra, A., *Lepra*, 1911–12, xii, 139.
13. Biehler, R., and Eliasberg, J., *Lepra*, 1909–10, ix, 207.
14. Lederman, R., *Lepra*, 1910, xi, 373.
15. Recio, A., *Lepra*, 1909–10, ix, 178.
16. Jeanselme, E., *Lepra*, 1912–13, xiii, 112.
17. Babes, V., and Busila, V., *Compt. rend. Soc. biol.*, 1909, lxvii, 817.
18. Babes, V., and Busila, V., *Compt. rend. Soc. biol.*, 1910, lxviii, 181.
19. Babes, V., and Busila, V., *Compt. rend. Soc. biol.*, 1910, lxix, 91.
20. Wills, F. F., *Centr. Bakt., Ite Abt., Orig.*, 1912, lxi, 37.
21. Clegg, M. T., McCoy, G. W., and Hollmann, H. T., *Pub. Health Bull., U. S. P. H., No. 66*, 1914.

(*b*) *Extracts of Normal Tissue, Syphilitic Tissue, and Tissue from Rat Leprosy.*

22. Wechselmann and Meier, G., *Lepra*, 1909, viii, 226.
2. Eitner, E., *Wien. klin. Woch.*, 1908, xxi, 729.
23. Slatinéanu, A., and Daniélopolu, D., *Compt. rend. Soc. biol.*, 1908, lxv, 347.
24. Masslakowetz, P. P., and Liebermann, J. J., *Centr. Bakt., Ite Abt., Ref.*, 1909, xliv, 490.
25. Jundell, Almkvist, J., and Sandman, F., *Centr. inn. Med.*, 1908, xxix, 1181.
26. Bruck, C., and Gessner, E., *Berl. klin. Woch.*, 1909, xlvi, 589.
27. Eliasberg, J., *Deutsch. med. Woch.*, 1909, xxxv, 1922.
3. Frugoni, C., and Pisani, S., *Berl. klin. Woch.*, 1909, xlvi, 1530.
28. de Azua, J., and Covisa, J. S., *Lepra*, 1910, ix, 143.
4. Thomsen, O., and Bjarnhjedinson, S., *Lepra*, 1909–10, ix, 191.
5. Nishiura, K., *Z. Immunitätsforsch., Orig.*, 1910, vii, 721.
29. Montesanto, D., and Sortiriadès, D., *Presse méd.*, 1910, xviii, 659.
6. Akerberg, H., Almkvist, J., and Jundell, I., *Lepra*, 1910, ix, 79.
30. Ehlers, K., and Bourret, G., *Lepra*, 1910, xi, 368.
7. Steffenhagen, K., *Berl. klin. Woch.*, 1910, xlvii, 1362.
31. Duval, C. W., and Gurd, F. B., *Arch. Int. Med.*, 1911, vii, 230.
32. Photinos, G., and Michaélidès, N., *Lepra*, 1911–12, xii, 207.
33. Nakajo, S., and Asakura, T., *J. Infect. Dis.*, 1915, xvii, 388.
8. Cooke, J. V., *J. Infect. Dis.*, 1919, xxv, 474.
12. Serra, A., *Lepra*, 1911–12, xii, 139.
16. Jeanselme, E., *Lepra*, 1912–13, xiii, 112.

34. Schmitt, L. S., *Univ. Calif. Pub. Path.*, 1911, ii, 29.
18. Babes, V., and Busila, V., *Compt. rend. Soc. biol.*, 1910, lxviii, 181.
35. Meier, *Lepra*, 1910, xi, 334.

(*c*) *Tuberculin and B. tuberculosis.*

36. Slatinéanu, A., and Daniélopolu, D., *Compt. rend. Soc. biol.*, 1908, lxv, 530.
3. Frugoni, C., and Pisani, S., *Berl. klin. Woch.*, 1909, xlvi, 1530.
7. Steffenhagen, K., *Berl. klin. Woch.*, 1910, xlvii, 1362.
33. Nakajo, S., and Asakura, T., *J. Infect. Dis.*, 1915, xvii, 388.
37. Slatinéanu, A., and Daniélopolu, D., *Compt. rend. Soc. biol.*, 1908, lxv, 528.
18. Babes, V., and Busila, V., *Compt. rend. Soc. biol.*, 1910, lxviii, 181.
19. Babes, V., and Busila, V., *Compt. rend. Soc. biol.*, 1910, lxix, 91.
38. Möllers, *Lepra*, 1914, xiv, 161.
20. Wills, F. F., *Centr. Bakt., 1te Abt., Orig.*, 1912, lxi, 37.

(*d*) *Acid-Fast Bacilli and Bacilli Isolated from Lepromas.*

3. Frugoni, C., and Pisani, S., *Berl. klin. Woch.*, 1909, xlvi, 1530.
7. Steffenhagen, K., *Berl. klin. Woch.*, 1910, xlvii, 1362.
31. Duval, C. W., and Gurd, F. B., *Arch. Int. Med.*, 1911, vii, 230.
8. Cooke, J. V., *J. Infect. Dis.*, 1919, xxv, 474.
9. Gaucher and Abrami, P., *Lepra*, 1909, viii, 152.
12. Serra, A., *Lepra*, 1911–12, xii, 139.
18. Babes, V., and Busila, V., *Compt. rend. Soc. biol.*, 1910, lxviii, 181.
19. Babes, V., and Busila, V., *Compt. rend. Soc. biol.*, 1910, lxix, 91.
38. Möllers, *Lepra*, 1914, xiv, 161.
39. Spronck, C. H. H., *Semaine méd.*, 1898, xviii, 393.
40. Mezincescu, D., *Compt. rend. Soc. biol.*, 1909, lxvi, 56.
20. Wills, F. F., *Centr. Bakt., 1te Abt., Orig.*, 1912, lxi, 37.
41. Kritschewsky, J., and Bierger, O., *Z. Hyg. u. Infectionskrankh.*, 1913, lxxiii, 509.
42. Currie, D. H., and Clegg, M. T., *Pub. Health Bull., U. S. P. H., No. 50*, 1911.
43. Wolbach, S. B., and Honeij, J. A., *J. Med. Research*, 1914, xxix, 367.
44. Clegg, M. T., *Philippine J. Sc.*, 1909, iv, 403.
45. Currie, D. H., Clegg, M. T., and Holmann, H. T., *Pub. Health Bull., U. S. P. H., No. 47*, 1911.
46. Duval, C. W., and Gurd, F. B., *J. Exp. Med.*, 1911, xiv, 181.
47. Duval, C. W., and Wellman, C., *J. Infect. Dis.*, 1912, xi, 116.
48. Duval, C. W., and Harris, W. H., *J. Med. Research*, 1913, xxviii, 165.
49. Harris, W. H., and Lanford, J. A., *J. Infect. Dis.*, 1913, xiii, 301.
50. Harris, W. H., and Lanford, J. A., *J. Med. Research*, 1916, xxxiv, 157.
51. Bayon, H., *Tr. Soc. Trop. Med.*, 1911–12, v, 158.
52. Bayon, H., *Brit. Med. J.*, 1912, ii, 1191.
53. Cooke, J. V., *J. Infect. Dis.*, 1919, xxv, 452.
54. Petroff, S. A., *Am. Rev. Tuberc.*, 1918, ii, 523.

55. Aronson, J. D., and Lewis, P. A., *Am. Rev. Tuberc.*, 1922–23, vi, 1024.
56. Kritschewsky, J. L., *Z. Immunitätsforsch., Orig.*, 1913–14, xx, 238.
57. Lewis, P. A., *Am. Rev. Tuberc.*, 1919, iii, 129.
21. Clegg, M. T., McCoy, G. W., and Hollmann, H. T., *Pub. Health Bull., U. S. P. H., No. 66*, 1914.

(*e*) *Unrelated Bacteria* (*B. typhosus, B. friedländeri, etc.*).

8. Cooke, J. V., *J. Infect. Dis.*, 1919, xxv, 474.
9. Gaucher and Abrami, P., *Lepra*, 1909, viii, 152.
16. Jeanselme, E., *Lepra*, 1912–13, xiii, 112.
41. Kritschewsky, J., and Bierger, O., *Z. Hyg. u. Infectionskrankh.*, 1913, lxxiii, 509.